THE ROLE OF THE POLY(A) SEQUENCE IN MAMMALIAN MESSENGER RNA

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I. INTRODUCTION

The occurrence of poly(A) as a component of eukaryotic messenger RNA has been known for about 10 years, but the role of this sequence is still something of a mystery. The poly(A) segment, located at the 3′ end of the RNA chains, and separated from the coding sequence by a long polynucleotide stretch, is apparently not needed for the translation process. It is added to newly synthesized nuclear RNA chains at an early stage of their conversion to mature mRNA molecules. After appearance of the mRNA in the cytoplasm, the poly(A) sequence becomes the target of a degradation process that causes a gradual reduction in its size. This process apparently does not lead to the accumulation of mRNA chains from which this sequence is missing. Thus it appears that a poly(A) segment of varying length accompanies the mRNA throughout its life cycle. Some mRNA species, however, do not normally possess a poly(A) sequence.

The poly(A) degradation process has received much attention. It would seem *a priori* that such an alteration of mRNA structure represents a maturation process that somehow modifies its function in the cell. The characteristics of newly synthesized and steady-state mRNA molecules have been compared, but no obvious correlation between poly(A) size and mRNA function has been established so far. The steady-state mRNA, with the shorter segments, appears to be as stable and as efficient in translation as the newly synthesized chains. The histone mRNAs, which lack poly(A) altogether, are also quite stable, although their degradation seems to be regulated in a distinct manner.

Much work has been carried out to determine the possible involvement of the poly(A) sequence in the events that lead to the conversion of primary transcripts to functional mRNA molecules. While most of the newly synthesized RNA is destroyed in the nucleus, the segments that acquire a poly(A) sequence are largely retained as mRNA. Thus poly(A) addition may be an essential part of the selection process. It is also possible that at least some of the processing events require the presence of the poly(A) segment.

The various experimental approaches to the study of poly(A) function have provided much significant information, but it has not yet been possible to fit the facts into a coherent picture. Some of the findings seem contradictory and confusing, and conclusions derived from early studies may need reevaluation in the light of more recent information. It may well be that the poly(A) sequence has more than one function. If this were the case, then attempts to fit all well-established facts into a single model would be self-defeating. This article is designed to discuss various findings that may

be relevant to the understanding of poly(A) function. It is not meant to provide a comprehensive review of the field, and only representative studies are used as a basis for discussion. The article deals primarily with studies of mammalian systems.

II. RELATION OF POLY(A) SEQUENCE TO mRNA STRUCTURE

A. Noncoding Sequences in mRNA

The primary structure of several eukaryotic mRNA species has been elucidated in recent years. In all cases studied so far a large portion of the RNA chain is occupied by sequences not involved in coding for amino acids. A relatively short polynucleotide sequence precedes the initiation codon, and a longer sequence follows the translation-termination signal. The poly(A) sequence is adjacent to the 3′ noncoding region, and terminates the mRNA chain.

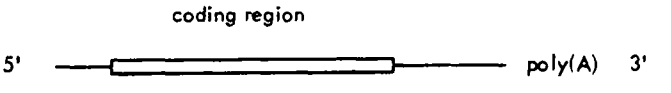
The occurrence of a long polynucleotide sequence past the termination codon was first indicated by the finding of a human hemoglobin variant (Hemoglobin Constant Spring) in which the α-chain had about 30 additional amino acids on the C-terminal side.[1] This mutation was traced to a base substitution in the normal termination signal, thus leading to read-through. Partial sequence determination of the human α-globin mRNA indicates that the 3′ noncoding region contains 112 nucleotides, and accounts for the amino acid sequence of the Constant Spring variant.[2] The length of this region in the rabbit and human globin mRNAs ranges between 89 and 135 nucleotides (Figure 1). This represents 14 to 22% of the total RNA chain, excluding the poly(A) sequence. The 3′ noncoding region is considerably larger in a mouse immunoglobulin light chain mRNA, and is larger still in chick ovalbumin mRNA where it accounts for 34% of the whole mRNA.

B. Poly(A) as 3′ Terminal Segment

Studies of the total mRNA population of mouse and human cells have also placed the poly(A) sequence at the 3′ end of the RNA chains. The general approach has been to determine whether this sequence is terminated by an AMP residue with a free 3′-OH. Treatment of the RNA with pancreatic ribonuclease released the poly(A) as segments terminated in this fashion. A poly(A) sequence located either internally or at the 5′ end of the RNA would have been released as a segment terminated by a phosphorylated 3′-OH group. Alkaline hydrolysis of the isolated poly(A) segments generates an adenosine residue from the 3′ terminal nucleotide (Figure 2). The poly(A) from cells labeled with radioactive adenosine yielded 1 nucleoside per about 200 adenylic acid (AMP) residues.[8] Since the size of newly synthesized poly(A) is around 200 nucleotides, the adenosine yield after alkaline hydrolysis indicates that essentially all the poly(A) segments in the cytoplasm are normally terminated by a free 3′-OH. In a different approach, the mRNA was treated with an exonuclease that attacks chains terminated by a free 3′-OH. This caused rapid hydrolysis of the poly(A) sequence.[9] Pretreatment of isolated poly(A) segments with alkaline phosphatase did not accelerate the exonuclease digestion.[10] This indicated that poly(A) segments with a phosphorylated 3′ terminus did not occur in the preparations.

C. Interaction of Poly(A) With Other Sequences

The studies of primary structure show that the poly(A) segment is far removed from the initiation site for translation. In the individual mRNA species characterized so far, it is also well separated from the entire coding region. This arrangement, however, does not preclude the possibility of noncovalent interactions between the poly(A) sequence and functional regions of the RNA chains. There is as yet no concrete infor-



α-globin, rabbit	36	426	89	(3)
α-globin, human	37	426	112	(2)
β-globin, rabbit	53	441	95	(4, 5)
β-globin, human	50	441	135	(2, 5)
immunoglobulin light chain, mouse			211	(6)
ovalbumin, chick	64	1158	637	(7)

FIGURE 1. Linear structure of eukaryotic mRNAs. Values represent size of coding and noncoding regions, expressed as number of nucleotides. Values in parenthesis are references.

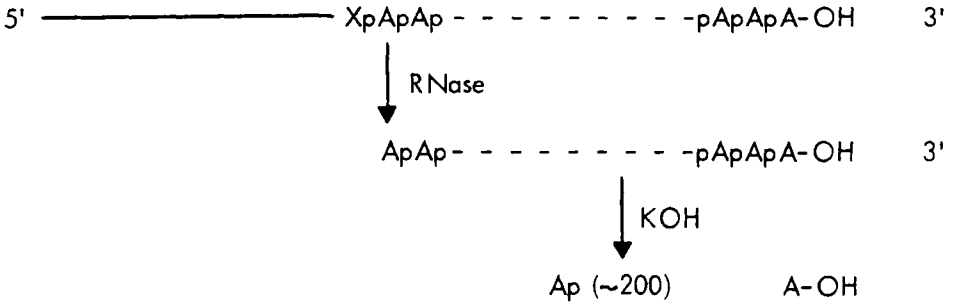


FIGURE 2. Localization of poly(A) sequence at 3′ end of mRNA and determination of number of AMP residues in sequence. Ap stands for 3′-AMP and A-OH stands for adenosine.

mation concerning specific interactions within the mRNA molecule. Moreover, these molecules are associated with proteins, and their overall conformation is likely to be affected by specific protein-polynucleotide interactions. For instance, the poly(A) sequence is associated with proteins (see Section I.F.) and it is not known whether this kind of arrangement would prevent or enhance its interaction with other polynucleotide segments. The possibility for the occurrence of such interactions has been examined by subjecting isolated polysomes to limited nuclease digestion, and isolating poly(A)-containing fragments after deproteinization of the digest.[11] The material bound to oligo(dT)-cellulose was found to contain polynucleotide fragments associated with the poly(A). These fragments have not been characterized, and the possibility of nonspecific associations being generated during the manipulations was not completely eliminated in that study.

A structural model has been proposed in which the poly(A) segment is linked to the ribosomal binding site by a small RNA chain that contains regions complementary to both ends.[12] This model was used to explain the inhibitory effect of small RNA molecules on the translation of selected mRNA species in embryonic muscle. There is, however, no evidence for such a structure, and the presumed linker RNA remains poorly characterized.

D. Length of Poly(A) Sequence

Poly(A) is synthesized in the nucleus as a sequence of uniform size. After transfer of the mRNA molecules to the cytoplasm, this sequence becomes the target of a degradation process which causes a gradual decrease in its size. The size distribution of poly(A) segments isolated from RNA molecules can be visualized by polyacrylamide gel electrophoresis. The newly synthesized nuclear poly(A) is seen to have a narrow distribution of sizes (Figure 3). Size measurements based on mobility relative to that of polynucleotides of known length have yielded values as high as 300 nucleotides for the nuclear poly(A) of mammalian cells.[14] A value of about 200 nucleotides was obtained by comparing the yields of adenosine and AMP after alkaline hydrolysis.[8] However, the latter estimates did not take into consideration the turnover that occurs at the end of the poly(A) sequences (see Section V.B.). When corrections were made to account for this turnover, a minimum value of 260 nucleotides was obtained for the average length of the poly(A) in the nucleus.[13]

The newly synthesized poly(A) in the cytoplasm is as homogeneous as its nuclear counterpart, but a small reduction in size is apparent after labeling periods as short as 5 min (Figure 3). The steady-state poly(A) has been studied by using cells labeled for extended periods,[14-16] or by assaying for the unlabeled poly(A) through annealing with radioactive poly(U).[14] Both approaches have shown the cytoplasmic poly(A) to be highly heterogeneous in size, with most of the segments being considerably shorter than the nuclear segments (Figure 3).

The size distribution of the poly(A) sequence in individual mRNA species has been studied. Highly purified chick ovalbumin mRNA yielded poly(A) segments with a wide distribution of sizes.[17] The absolute values were reported to be in the 30 to 100 nucleotide range. The poly(A) sequences in globin mRNA also have proved to be heterogeneous in size. The poly(A) of globin mRNA from mouse reticulocytes, analyzed by gel electrophoresis, showed the existence of at least two size classes.[18,19] Two distinct peaks were also observed when the poly(A) of rabbit globin mRNA was subjected to zone centrifugation.[20] The different size classes observed in the preparations from mouse reticulocytes are apparently present in both the α- and β-globin mRNAs.[18,19] The absolute values reported from different laboratories for the sizes of the poly(A) sequence in globin mRNA vary quite widely. Values as low as 10 to 30 nucleotides have been obtained for the steady-state poly(A) of circulating rabbit reticulocytes.[21] The differences may be due in part to the use of reticulocytes at different stages of maturation as source of the mRNA. Also the lack of reliable size markers makes it difficult to make accurate estimates of poly(A) size.

The above studies indicate that individual mRNA species have poly(A) sequences that can vary widely with respect to size. They leave unanswered the question whether different mRNA species can have distinct poly(A) size distributions. The steady-state poly(A) segments of rabbit globin mRNA have been compared to those of total mouse ascites cell mRNA, and found to be considerably shorter (Figure 4). The significant factor in this comparison, however, is most probably the physiological state of the cells, and not the nature of the mRNA species. The reticulocytes, having lost the ability to synthesize RNA, contain only "old" mRNA molecules, while the ascites cells have a spectrum of molecules at varying stages of maturation.

Another approach has been used to compare the poly(A) size distribution in individual mRNA species. Segments of different sizes can be distinguished by appropriate binding procedures. Adsorption on Millipore® filters in the presence of high salt is effective only for segments of relatively large size, probably longer than 50 nucleotides.[19] Oligo(dT)-cellulose can bind shorter poly(A) segments, probably down to about 15 nucleotides in size;[22] and poly(U)-sepharose binds still shorter segments.[23]

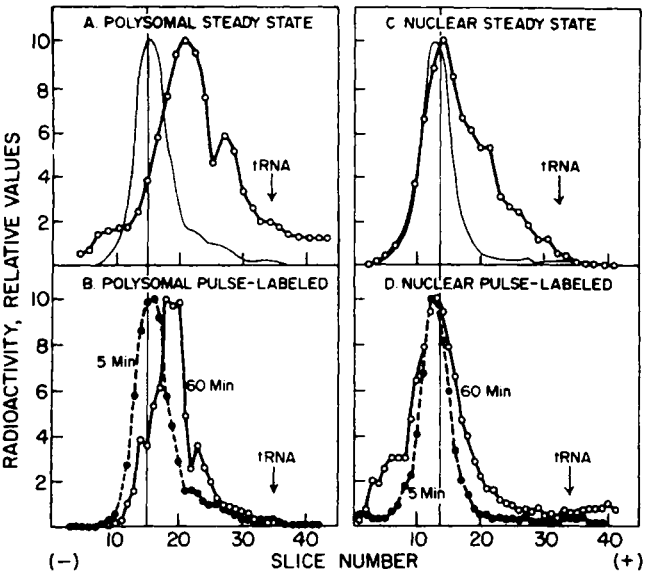


FIGURE 3. Polyacrylamide gel electrophoresis of newly synthesized and steady-state poly(A) of mouse sarcoma cells. Nuclear and cytoplasmic poly(A) preparations from unlabeled cells, or from cells labeled for specified time periods, were subjected to polyacrylamide gel electrophoresis. Thin vertical lines indicate position of mobility 0.4 relative to that of a tRNA marker. Profiles with thin lines in upper portion of diagram represent poly(A) labeled for 5 min redrawn for comparison. Data plotted on relative scale of 10 for better comparison of profiles. (Reproduced from Brawerman, G. and Diez, J., *Cell*, 5, 272, 1975. With permission.)

This approach permits the analysis of poly(A) size distribution in functional mRNA species. The preparations bound to different adsorbents are translated in a cell-free system, and the distribution of specific polypeptides among the translation products is examined. This analysis has shown that the majority of the abundant mRNA species of mouse sarcoma ascites cells have primarily chains with poly(A) sequences large enough to bind to Millipore®, and an insignificant proportion of chains unable to bind to oligo(dT)-cellulose. The mRNA for actin, however, seems to have mostly chains with relatively short poly(A) sequences unable to bind to Millipore®.[24] Moreover, a substantial proportion of the actin mRNA of various mammalian cells cannot bind to oligo(dT)-cellulose, but is still capable of binding to poly(U)-sepharose.[23,24] This indicates that the mRNA for nonmuscle actin has a distinct distribution of poly(A) sizes, with a preponderance of chains with segments smaller than 50 nucleotides in length (Table 1).

Messenger RNA can also be fractionated on the basis of poly(A) size by stepwise elution from poly(U)-sepharose, using either increasing formamide concentrations[25] or increasing temperatures.[26] This approach has been applied to the study of two adenovirus mRNA species from transformed rat cells that differ with respect to metabolic stability.[25] In this case, the individual mRNAs were assayed by annealing labeled preparations to specific DNA probes. Differences in poly(A) size distribution were observed between the two species, and also between the viral mRNAs and the total cellular mRNA (Figure 5). This study was performed with RNA preparations labeled for increasing time periods, and the newly synthesized poly(A) sequences of the individual mRNA species were seen to decrease in size at different rates (see section V.D.). The

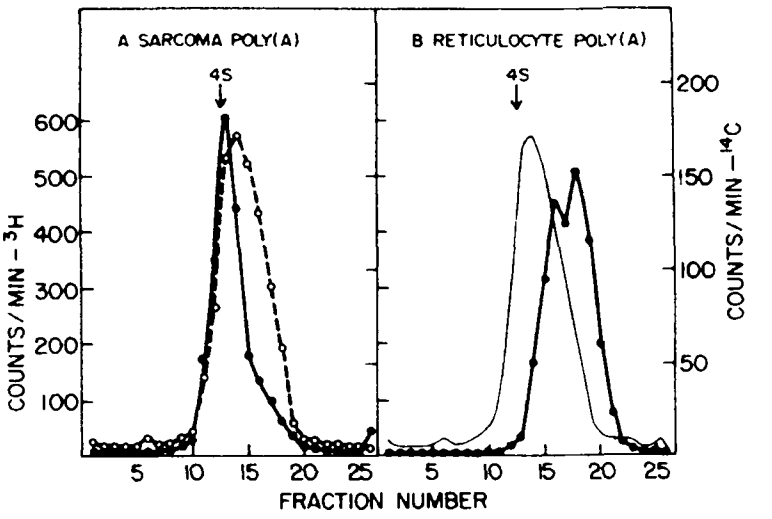


FIGURE 4. Zone sedimentation of poly(A) from sarcoma and reticulocyte polysomal RNA. RNase-treated material was deproteinized and centrifuged through sucrose gradients in 50 m*M* Tris-HCl (pH 7.6), 100 m*M* KCl, and 5 m*M* MgCl, for 40 hr at 40,000 r/min in the Spinco SW41 rotor. (A) Comparison of sedimentation profiles of newly synthesized poly(A) and steady-state poly(A) from sarcoma polysomal RNA. (—) ^{14}C-Adenine polysomal RNA used in order to permit comparison of newly synthesized and total poly(A); (---) distribution of total poly(A) as determined by ^{3}H-poly(U) assay on separate aliquots of each gradient fraction. (B) Comparison of sedimentation profiles of steady-state poly(A) from reticulocyte and sarcoma polysomes. Sedimentation profile of steady-state sarcoma poly(A) from panel A is reproduced (light line) for comparison. Position of 4S marker was determined by centrifugation of *E. coli* tRNA in a separate tube. (Reproduced from Jeffery, W. R. and Brawerman, G., *Biochemistry*, 13, 4636, 1974. With permission.)

Table 1
BINDING OF mRNA FOR ACTIN TO MILLIPORE® FILTER AND TO OLIGO(dT)-CELLULOSE[24]

Polypeptide synthesized	Activity in total RNA	Activity in nonadsorbed fractions	
		Millipore® filter	Oligo(dT)-cellulose
Actin	150	87	45
P-50	40	10	

Note: Sarcoma-180 polysomal RNA was adsorbed to oligo(dT)-cellulose. An identical RNA sample was passed through a Millipore® filter in the presence of 500 m*M* KCl, 1 m*M* MgCl, and 10 m*M* Tris-HCl (pH 7.6). Amounts of total RNA and unadsorbed RNA fractions used to direct translation in vitro were 4 μg. Translation products were subjected to polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$ and urea. Values are expressed as amounts of polypeptide synthesized (in arbitrary units) per microgram of added RNA. P-50 represents a major polypeptide of 50,000 mol wt that is coded by an mRNA with binding characteristics similar to those of the bulk of the cellular mRNA.

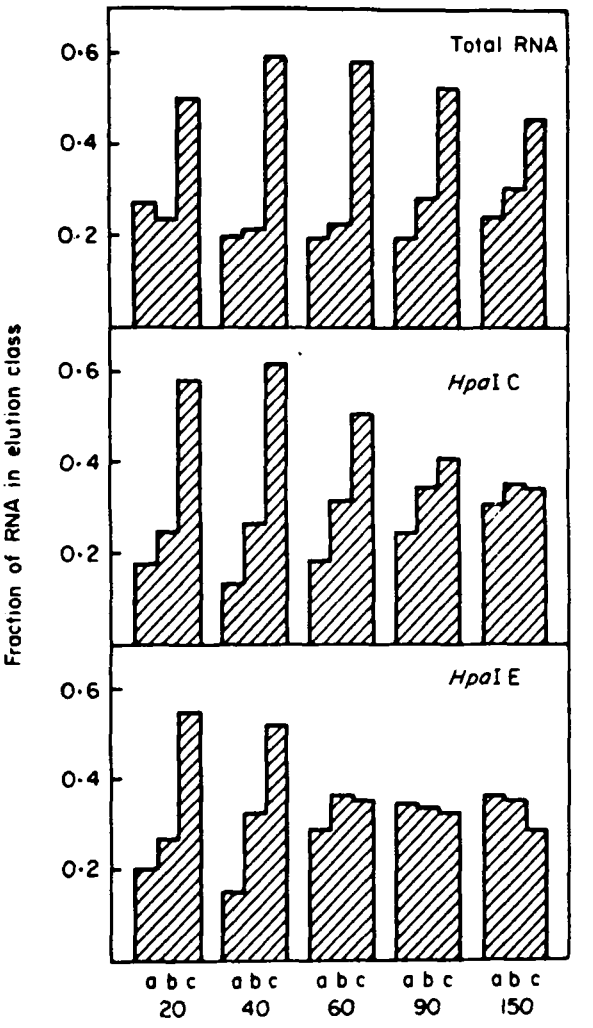


FIGURE 5. *Fractionation of AD2-specific RNAs by poly(A) length during continuous labeling.* Cells were labeled with ^{3}H-uridine. Samples were removed at the indicated times and total cytoplasmic RNA was extracted. The RNA was applied to poly(U)-sepharose columns and the mRNA eluted at formamide concentrations of 20%, 25%, and 35% (v/v) was analyzed for AD2-specific sequences by hybridization. The incorporation into hybrid molecules increased from a total of (*HpaI* E) 681 cpm to 6729 cpm and (*HpaI* C) 544 cpm to 16,328 cpm during the labeling from 20 to 150 min. A portion of each fraction was also taken to measure the radioactivity in total acid-precipitable mRNA. The data are presented as histograms showing the proportion of total or Ad2-specific mRNA molecules containing poly(A) lengths of (a) 50 nucleotides or less, (b) 50 to 100 nucleotides, and (c) 100 to 200 or more nucleotides. (Reproduced from Wilson, M. C., Sawicki, S. G., White, P. A., and Darnell, J. E., Jr., *J. Mol. Biol.*, 126, 31, 1978. With permission.)

measurements were carried out with mRNA labeled for no more than 2.5 hr and it is not known, therefore, to what extent the corresponding steady-state mRNA populations may differ with respect to poly(A) size.

E. Association of the Poly(A) Sequence With Protein

Whatever function the poly(A) sequence may have in the cell must be mediated by interactions between this sequence and other components. Such interactions may be limited, or modulated, by proteins that are associated with the poly(A). The presence of proteins on this sequence was demonstrated by subjecting polyribosomes and mRNP particles to nuclease digestion. The poly(A) segments released in this fashion sediment as 8- to 12S components in contrast to the 3 to 4S sedimentation coefficients of segments released from deproteinized RNA.[20,27] The poly(A)-protein complex that bears newly synthesized poly(A) is more homogeneous, with a sedimentation coefficient of 12S (Figure 6). The components with the smaller sedimentation values were shown to carry the shorter poly(A) segments.[28] This was also observed in the author's laboratory. The poly(A) complex derived from rabbit reticulocyte polysomes resembles the smaller ascites cells complexes. It sediments as a relatively homogeneous 8S component.[20]

The nature of the material associated with the poly(A) is still a matter of some controversy. Pronase digestion of the isolated complex releases free poly(A).[27] It has been reported that a single polypeptide with mol wt 78,000 is associated with the poly(A) segments released from polysomes by ribonuclease digestion.[29] This analysis, however, was carried out on a complex of unknown purity, and no data on relative amounts of poly(A) and protein were provided. A more detailed analysis[28] showed a variety of polypeptides to be present in the poly(A)-protein peak obtained by zone centrifugation of nuclease-treated mRNP complexes. A polypeptide of 75,000 daltons was present only among the larger poly(A)-protein complexes. Treatment of the cells with 3′-deoxyadenosine, which blocks the appearance of newly synthesized mRNA into the cytoplasm (see Section VI.D.) caused a sharp reduction in the amount of this protein. It was suggested by the authors that the 75,000-dalton polypeptide is present only on the poly(A) sequence of newly synthesized mRNA. The studies of protein associated with mRNA have been hampered by the lack of effective purification procedures. Definitive information on the proteins associated with the poly(A) sequence will require the availability of highly purified preparations.

F. Masking of the Poly(A) Sequence in Nucleoprotein Complexes

Because of its association with protein, the poly(A) sequence is well protected against the action of some nucleases. It has been possible to study the nature of the poly(A)-protein interaction by measuring the extent of protection under various conditions. Incubation of intact polysomes or mRNP particles with snake venom exonuclease results in little, if any, poly(A) degradation.[30] The poly(A) of deproteinized RNA is degraded rapidly under these conditions (Figure 7). A similar effect is observed with polynucleotide phosphorylase, but only when the reaction is carried out in the cold.[31] A cytoplasmic extract of mouse ascites cells is capable of causing poly(A) degradation in the polysomes, but at a rate slower than free poly(A) hydrolysis (Figure 7). The activity of the extract was attributed to a soluble factor that renders the poly(A) sensitive to nuclease.[30] The factor remains to be characterized and its mode of action better defined.

The poly(A)-protein interaction appears to be dependent on the integrity of the mRNA chain. Fragmentation of the polysomes by pancreatic RNase renders this sequence sensitive to snake venom exonuclease (Table 2). This is not due to loss of the protein component, since the endonuclease treatment releases the poly(A) as a nucleoprotein complex. Studies with the isolated complex have shown that the poly(A) is still protected in this state, but that it becomes increasingly sensitive to the enzyme upon incubation at 37°C.[89] This behavior suggests that the poly(A)-protein interaction may be reinforced, and perhaps modulated, by some other portion of the mRNA.

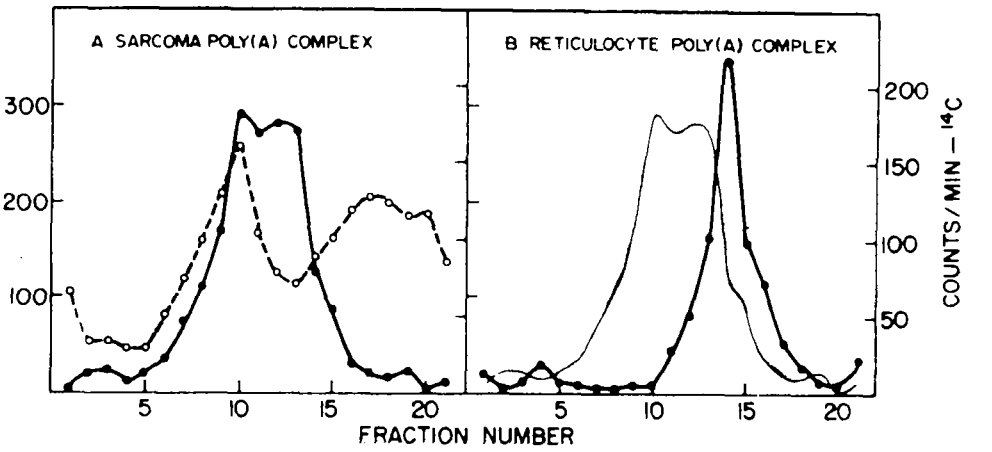


FIGURE 6. Zone sedimentation of the poly(A)-protein complexes from sarcoma and reticulocyte polysomes. Two A_{260} units of polysomes were digested with RNase and centrifuged through sucrose gradients for 18 hr at 35,000 r/min in the Spinco SW50 rotor. (A) Comparison of sedimentation profiles of newly synthesized and steady-state poly(A) complex from sarcoma polysomes. Polysomes labeled with ^{14}C-adenine were used in order to permit comparison of newly synthesized (---) and total (—) poly(A)-complex. Distribution of total poly(A) was determined by the ^{3}H poly(U) assay on separate aliquots of each fraction. (B) Comparison of sedimentation profiles of steady-state poly(A)-protein complexes from reticulocyte and sarcoma polysomes. (—) Distribution of reticulocyte poly(A) as determined by ^{3}H-poly(U) assay. Sedimentation profile of steady-state sarcoma poly(A)-protein complex from panel (A) is reproduced (light line) for comparison. Position of 4S marker is at fraction number 18. (Reproduced from Jeffery, W. R. and Brawerman, G., *Biochemistry*, 13, 4635, 1974. With permission.)

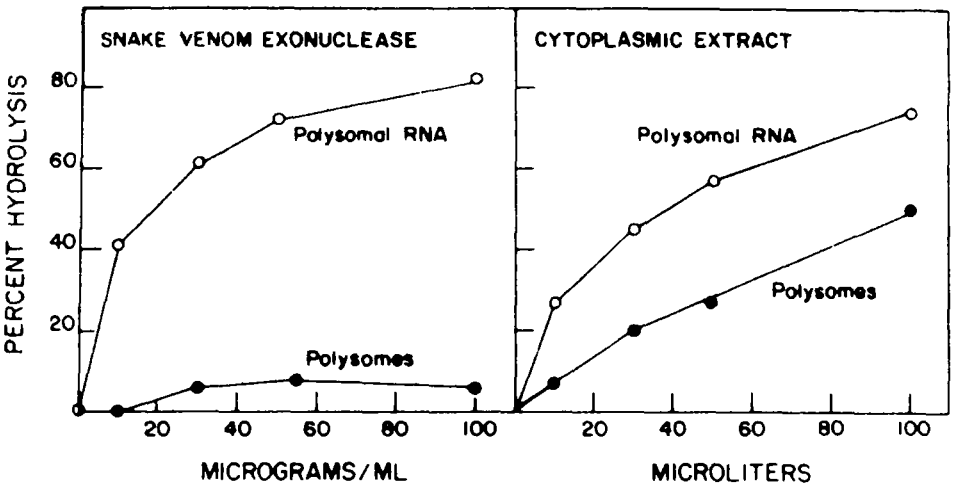


FIGURE 7. Effect of snake venom exonuclease and of cytoplasmic extract from ascites cells on poly(A) degradation in polysomes and in polysomal RNA. Mixtures of adenosine-labeled polysomes and unlabeled RNA, or of adenosine-labeled RNA and unlabeled polysomes, were incubated at 37°C for 30 min, the presence of either exonuclease or crude cytoplasmic extract. The concentration of both labeled and unlabeled components was 0.9 A_{260} units/m*l* of incubation mixture. Values expressed as percent of radioactivity in poly(A) or controls incubated under identical conditions without extract or snake venom enzyme. (Reproduced from Bergmann, I. and Brawerman, G., *Biochemistry*, 16, 260, 1977. With permission.)

Table 2
EFFECT OF PANCREATIC RIBONUCLEASE ON POLY(A) DIGESTION BY EXONUCLEASE[30]

	Poly(A) digestion		
RNase A	No addition	Exonuclease	RNA digestion
None	—	1	
0.1 μg	1	13	46
0.5 μg	2	19	48

Note: Labeled polysomes incubated for 10 min with 5 μg/ml of snake venom exonuclease in presence or absence of indicated amounts of pancreatic RNase A. Extent of RNA digestion in polysomes determined by precipitation with cold trichloroacetic acid. Values are expressed as percent hydrolysis.

The protection against exonuclease indicates that the 3′ terminus of the poly(A) sequence interacts strongly with the protein component. The presence of protein on this sequence also prevents its degradation by endonucleases, such as bovine pancreatic RNase[28] and chick oviduct nuclease.[32] Exposure of the isolated complex to RNase T_2 results in relatively slow hydrolysis, which is probably due to gradual destabilization during the incubation.[27] Micrococcal nuclease has a similar effect. Thus the entire length of the poly(A) sequence appears to be covered with protein.

The extent of masking of the poly(A) sequence can also be studied by measuring its ability to form stable base-paired structures with radioactive poly(U). This reaction has been used to assay for steady-state poly(A) in nucleoprotein particles.[20,27] Incubation of poly(U) with these particles under annealing conditions does produce RNase-resistant structures, but the yields are considerably lower than those obtained with equivalent amounts of deproteinized RNA.[89] Also, the poly(U)-containing structures obtained in this fashion may not represent genuine poly(A)-poly(U) duplexes or triplexes, since the reactions differ to some extent with respect to ionic requirements.

The above studies indicate that the proteins associated with the poly(A) sequence can restrict its ability to interact with other polynucleotides and with nucleases. It remains to be determined to what extent these interactions affect the behavior of the poly(A) sequence in vivo. It is known, for instance, that this sequence is subject to degradation in the cytoplasm. This would indicate that the bound proteins do not provide absolute protection in the intact cells.

G. Enzymatic Removal of Poly(A) From Polysomes

Studies with isolated polysomes have revealed another possible mechanism for the removal of the poly(A) sequence. While the latter is shielded from nucleases by protein components the adjacent mRNA segment appears to be particularly exposed. Limited digestion of purified polysomes with micrococcal nuclease causes release of the poly(A)-protein complex under conditions that cause relatively little fragmentation of the body of the mRNA.[90] Polysomes as they exist in freshly prepared cytoplasmic extracts are also cleaved in this fashion, simply by brief incubation of the extract. Messenger RNA chains depleted of poly(A), but still capable of promoting the synthesis of defined polypeptides, are generated by this treatment.

The preferential cleavage of the poly(A) sequence from polysomes may not seem surprising, since a large RNA segment not covered with ribosomes occurs next to it (see Section II.A.). This arrangement, however, could provide a basis for differential

susceptibility of individual mRNA species to poly(A) loss. The diversity of primary structure in the 3′ noncoding region could lead to differences in the degree of folding and in possible binding sites for protein, thus leading to varying degrees of exposure to intracellular nucleases.

III. MESSENGER RNA WITHOUT POLY(A) SEQUENCE

A. Characterization

The occurrence of mRNA species without poly(A) makes it difficult to formulate an essential role for this sequence. It is important, therefore, to assess the evidence for the notion that the occurrence of this type of RNA is widespread in eukaryotic cells. It is also important to determine whether it may have unique functional characteristics.

The criterion commonly used for the identification of poly(A)-lacking chains in mRNA preparations is failure to bind to oligo(dT)-cellulose. Messenger RNA chains with short poly(A) segments, however, may not be able to bind to this adsorbant, and can be mistaken for poly(A)-lacking molecules. The minimum required length for effective binding seems to vary with ionic conditions and temperature; it is probably in the range of 15 to 30 nucleotides. Poly(U)-sepharose can bind mRNA chains that fail to bind to oligo(dT)-cellulose.[23] On the other hand, it is questionable whether very short poly(A) segments are functional in the cell, and whether they are capable of carrying the proteins associated with this sequence. Thus it is conceivable that mRNA chains unable to bind to oligo(dT)-cellulose can be considered as lacking a functional poly(A) sequence (see Section IV.B.).

Another difficulty with the characterization of poly(A)-lacking mRNA is the potential for loss of this sequence during isolation of the RNA. It has been observed that phenol extraction can leave the poly(A) segment bound to denatured proteins, while the rest of the mRNA chain is released into the aqueous phase.[33] Recent studies indicate that a short incubation of cytoplasmic extracts can lead to preferential cleavage of the poly(A) segment (see Section II.G.). It is possible, therefore, that inadequate control of nucleases during RNA isolation may lead to recovery of functional RNA molecules from which the poly(A) sequence has been lost accidentally.

B. Histone mRNA

The histone mRNAs are identified as a broad 9S component in polysomal RNA preparations from pulse-labeled cells. Histone synthesis is linked to DNA replication, and is reduced considerably in cells inactive in this process.[34] The amount of 9S component is also reduced in these cells.[35,36] Very little poly(A) was found in the 9S peak of HeLa cell RNA preparations labeled with adenosine or with ^{32}P.[35] Hydroxylapatite fractionation of polysomal RNA annealed to poly(U), which separates poly(A)-containing from poly(A)-deficient RNA chains, left the 9S component in the latter fraction.[37] Sequence analysis of highly purified histone mRNA preparations from sea urchin has confirmed the absence of poly(A) from these RNA chains.[38]

The status of poly(A) in histone mRNA has become less clear with the detection of poly(A)-containing histone mRNA chains in amphibian oocytes,[39,40] and even in HeLa cells.[41] The RNA fraction of *Xenopus* oocytes that binds to oligo(dT)-cellulose was found to contain template activity for histone synthesis, although a large portion of the activity was still present in the unadsorbed fraction.[39] In a more detailed study, oligo(dT)-primed cDNA synthesized off poly(A)-containing RNA of *Triturus* oocytes was shown to anneal to a histone-specific DNA fragment.[40] No such cDNA was produced from the RNA fraction unable to bind to oligo(dT)-cellulose, even though the

latter fraction was active in promoting histone synthesis in a cell-free system. This is probably the most convincing evidence for the complete lack of poly(A) sequence from some of the histone mRNA chains.

A reasonable explanation has been offered for the occurrence of poly(A) in the histone mRNA of oocytes.[39] Histone synthesis is normally dependent on DNA replication, but it takes place in the absence of this process in amphibian oocytes. It could be that it is the lack of poly(A) that causes the histone mRNAs to decay when DNA synthesis is completed. These RNA species would then require a poly(A) sequence in order to remain functional in the oocytes. The occurrence of poly(A)-containing histone mRNA chains in HeLa cells, however, remains to be explained. It may be significant in this respect that some histone synthesis seems to continue in these cells after cessation of DNA synthesis.[42]

The origin of the two classes of histone mRNA is difficult to understand. It is unlikely that the predominant poly(A)-deficient chains arise through rapid loss of this sequence. It seems rather that the histone gene transcripts do not acquire any poly(A), since the latter is absent from the newly synthesized mRNA molecules. It is not known whether the poly(A)-containing histone mRNA is generated independently. It could be produced from mature chains lacking this sequence, since a process of poly(A) synthesis occurs in the cytoplasm (see Section V.B.).

C. Actin mRNA

Actin is produced in large amounts in a wide variety of mammalian cells. The greater portion of the template for actin synthesis is associated with the RNA fraction that binds to oligo(dT)-cellulose (Table 1), but a substantial proportion remains with the unadsorbed fraction, even after several cycles of binding.[23,24,43] Part of the latter material, however, can still bind to poly(U)-sepharose.[23] It appears, therefore, that the "poly(A)-deficient" actin mRNA, as defined by inability to bind to oligo(dT)-cellulose, represents a mixture of chains with very short segments and of chains that may be completely free of poly(A).

The different actin mRNA fractions seem to be part of the same steady-state population with a continuous distribution of poly(A) sizes. The average size of the poly(A) of actin mRNA is known to be relatively small (see Section II.D.). This could account for the fact that a substantial portion of the mRNA chains have poly(A) sequences too short to permit binding to oligo(dT)-cellulose. It has been shown that both fractions code for the β-actin isomer.[23,43] Thus it is likely that they are derived from the same actin gene.

It remains to be determined whether there exists a physiological difference between the actin mRNA chains with normal poly(A) and with very short poly(A). It is possible that poly(A) sequences of 15 to 20 nucleotides may be too short to be functional (see Section IV.B.). Thus the latter mRNA chains could be functionally similar to the poly(A)-lacking histone mRNA.

D. Cellular mRNA Populations

Various studies have been undertaken to determine how widespread poly(A)-lacking mRNA species may be. As a first approach, the nonribosomal RNA derived from polysome preparations has been examined. This RNA can be labeled selectively by using inhibitors of rRNA synthesis. This procedure, however, can lead to the labeling of a variety of RNA species. In order to improve the selectivity, isolated polysome preparations have been disrupted by EDTA. This causes the release of mRNA as nucleoprotein particles sedimenting in the 20 to 90S range. Presumably, only RNA originally associated with ribosomes is released into this fraction. About 30% of the la-

beled RNA obtained in this fashion from HeLa-cell polysomes was unable to bind to oligo(dT)-cellulose.[44] The latter material had sedimentation characteristics similar to those of the poly(A)-containing RNA.

Studies of translation of polysomal RNA in cell-free systems have provided more convincing evidence for the occurrence of a substantial population of mammalian mRNA chains unable to bind to oligo(dT)-cellulose. The distribution of abundant HeLa-cell mRNA species in the bound and nonbound fractions has been studied by comparing their translation products.[43] Most of the species present in the poly(A)-deficient (nonbound) fraction were also represented in the poly(A)-containing fraction (Figure 8). Only a few species seemed to be present exclusively in the poly(A)-deficient RNA fraction. Thus the majority of the mRNA species that occur in mammalian cells with very short poly(A) segments or with no poly(A) at all seem to belong to the same category as the actin mRNA. This would leave only a very limited number of species analogous to the histone mRNAs.

The above studies do not provide information on the vast population of minor mRNA components, since these cannot be detected through their translation products. Comparisons of total template activity in cell-free systems (measured by incorporation of radioactive amino acids into material insoluble in hot trichloroacetic acid), suggest that 20 to 30% of the cellular mRNA fails to bind to oligo(dT)-cellulose.[43,45] Only about 10 to 15% of the template activity flows through poly(U)-sepharose[90] but part of the latter activity could be due to fragmented mRNA chains still capable of initiating polypeptide synthesis. Thus the translation data suggest that a relatively small portion of the cytoplasmic mRNA molecules belong to the poly(A)-lacking class.

The available evidence concerning the poly(A)-lacking mRNA components seems somewhat contradictory. The labeling studies indicate that a large portion (about 30%) of the heterogeneous polysomal RNA is poly(A)-deficient, as judged by its inability to bind to oligo(dT)-cellulose.[43] This fraction also contains 30 to 40% of the total sequence diversity of polysomal RNA.[45a] Hybridization studies have shown that there is little overlap in the sequences of the poly(A)-containing and poly(A)-deficient polysomal RNA fractions.[44,45a] This indicates that the RNA species in the latter fraction represent distinct entities, not likely to be derived from poly(A)-containing RNA molecules through loss of this sequence. Translation of the mRNA in the poly(A)-deficient material, however, indicates that most of the abundant species in that fraction are also represented in the poly(A)-containing RNA. Cross-hybridization experiments do show some overlap among the abundant sequences of the two RNA fractions.[43] The vast population of less abundant poly(A)-lacking RNA species could represent a unique mRNA class, but the activity of this material in translation assays is rather low, particularly after passage through poly(U)-sepharose. In the absence of a functional test for this RNA fraction, its identification as poly(A)-lacking mRNA must be considered as tentative.

IV. EFFECT OF THE POLY(A) SEQUENCE ON mRNA BEHAVIOR

A. Translation Characteristics in Cell-Free Systems

The simplest approach to the study of poly(A) function is to compare the biological and biochemical properties of mRNA chains which contain this sequence to those which do not. It is possible to remove the poly(A) from purified mRNA preparations by digestion with polynucleotide phosphorylase, which functions as a 3′ exonuclease. The poly(A) segment of rabbit globin mRNA is apparently more sensitive to the enzyme than the rest of the mRNA chain. After limited digestion, a portion of the mRNA no longer binds the oligo(dT)-cellulose.[46] It sediments as a relatively homogeneous peak, somewhat more slowly than the treated mRNA that still binds to this adsorbent.

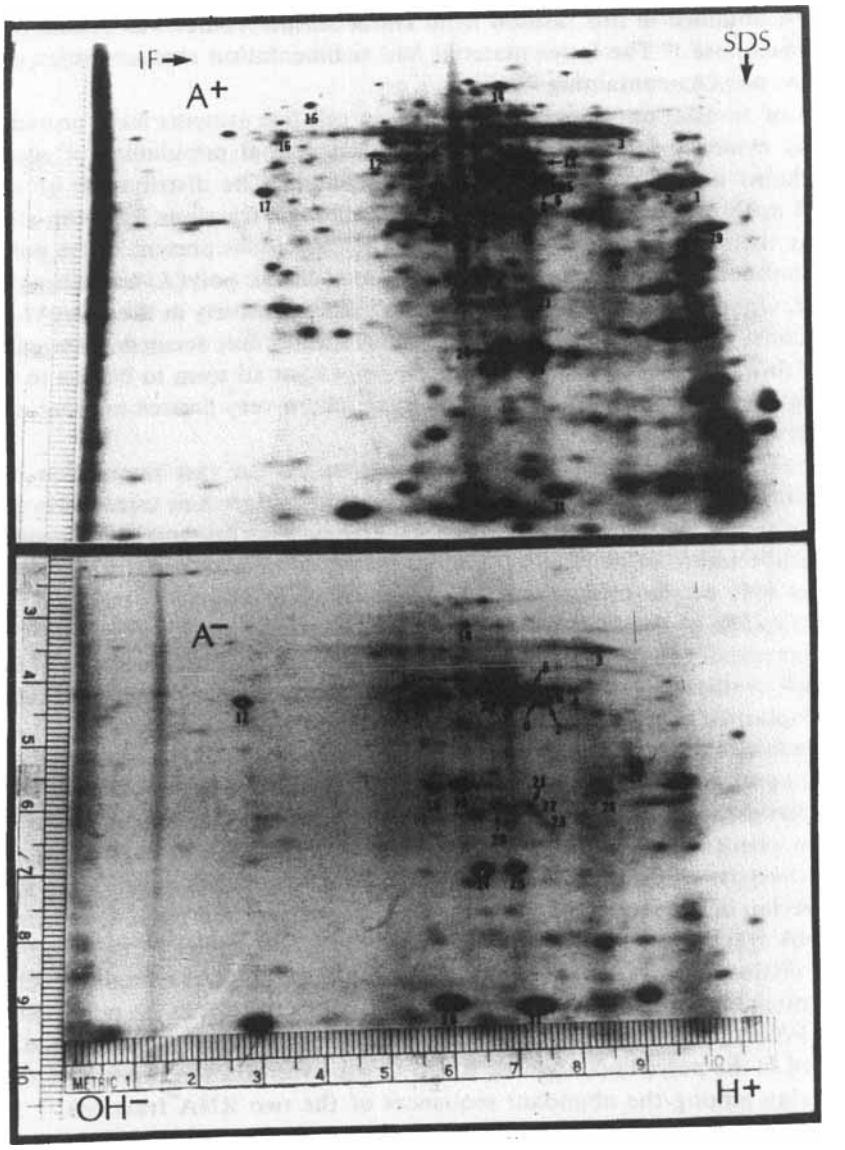


FIGURE 8. Fluorographs of two-dimensional gel electrophoresis of the poly(A)⁻ and poly(A)⁺ RNA in vitro translation products. Wheat germ S30 was used for translation of poly(A)⁻ RNA (lower) or an isolated poly(A)⁺ RNA (upper). A sample containing 15,000 cpm of poly(A)⁻ products (including 3000 cpm of background incorporation) or 30,000 cpm of poly(A)⁺ RNA products mixed with 3,000 cpm of background mixture was taken and 2 μg of unlabeled actin purified from HeLa cells or 1 μg of unlabeled actin from chicken thigh muscle was added to each sample. The first dimension was isoelectric focusing (1) and the second dimension was on sodium dodecyl sulfate/10 to 15% gradient polyacrylamide gels (2). The gels were stained, fluorographed, dried, and exposed to X-ray films for 21 days. Numbers refer to spots located above the numbers. Products 30 and 31 are synthesized in the background mixture. Unlabeled purified actin from HeLa cells coincided, on these gels, with product 9, and α form of actin was located between products 8 and 9. (Reproduced from Kaufmann, Y., Milcarek, C., Berissi, H., and Penman, S., Proc. Natl. Acad. Sci. U.S.A., 74, 4801, 1977).

The poly(A)-deficient globin mRNA fraction was still capable of promoting globin synthesis in an ascites cell-free system.[46] When the yields of total polypeptide synthesized as a function of added mRNA were compared, the loss of the poly(A) sequence seemed to have no effect on the efficiency of the mRNA. The poly(A)-deficient fraction, however, was considerably less effective in promoting the synthesis of globin. It is not known whether this reduced efficiency is due solely to loss of the poly(A) sequence, since the extent of enzymatic digestion was not rigorously controlled.

In a similar study, involving the use of a highly purified enzyme and strictly controlled conditions of digestion, the poly(A)-deficient globin mRNA was found to be as effective as the intact mRNA chains in promoting globin synthesis in the ascites cell-free system.[31] A close examination of the time course of the reaction, however, suggested that the mRNA depleted of poly(A) was losing its activity more rapidly (Figure 9), as if this sequence were protecting the mRNA from degradation during the incubation.

A study of chick oviduct mRNA depleted of poly(A) led to different conclusions. The treated mRNA seemed to be less efficient in the initiation process.[47] The size of the polysomes generated in the cell-free system was somewhat smaller, and the yield of labeled ovalbumin as a function of input RNA was also reduced. These effects were observed only in the reticulocyte cell-free system. The two types of ovalbumin mRNA were undistinguishable in the wheat germ cell-free system. The difference in response was attributed to the fact that the reticulocyte lysate has a greater capacity for polypeptide chain initiation. This would make it more responsive to changes in the initiation efficiency of the added mRNA. No effect of poly(A) removal on the apparent stability of the mRNA was observed in this study.

The above studies were based on the expectation that the enzyme treatment would not reach beyond the poly(A)-sequence. While endonucleolytic cleavage can be detected through size measurements, the removal of some nucleotides from the 3′ end of the transcribed region cannot be measured easily. The enzymic digestion of the ovalbumin mRNA was shown not to reduce the capacity of the RNA to anneal with ovalbumin cDNA.[47] The latter test, however, does not seem adequate to detect a small loss from the 3′ noncoding region. It would seem that more rigorous controls are needed to show clearly that the alterations in mRNA properties are due to loss of poly(A).

A different procedure, more selective in principle, has been used to remove the poly(A) sequence. The mRNA preparations are annealed to poly(dT) and the resulting hybrids are digested with RNase H, specific for DNA-RNA hybrids. Since only the poly(A) sequence is in hybrid form in the annealed preparations, the treatment should destroy this sequence selectively. Globin mRNA treated in this fashion proved to be as effective as the intact RNA in promoting globin synthesis in several cell-free systems.[48] The reticulocyte cell-free system, however, was not used in that study. There appeared to be an effect on the stability of the mRNA. Preincubation in the presence of an ascites cell-free system caused somewhat more rapid inactivation of the mRNA depleted of poly(A). A more effective test for measuring initiation efficiency was also used. The intact and treated globin RNAs were compared with respect to their ability to compete against intact ovalbumin mRNA. Ovalbumin synthesis was inhibited to the same extent by the two globin mRNA preparations. Unfortunately, globin synthesis was not measured under these conditions of competition, and it is not clear that the experiment did in fact assess the initiation efficiency of the globin mRNAs.

The naturally occurring poly(A)-containing and poly(A)-deficient forms of actin mRNA have been compared with respect to translation efficiency in the wheat germ system.[24] In this case, the effect of free poly(A) or poly(U) on the efficiency of trans-

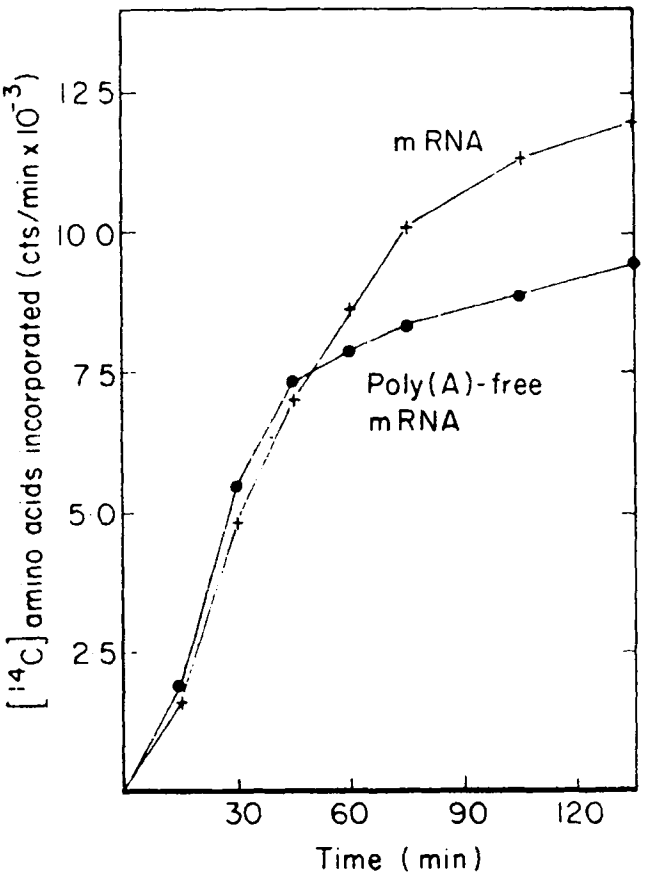


FIGURE 9. Time course of protein synthesis in the ascites tumor cell-free extract. The mRNA concentrations used were 0.53 μg mRNA or 0.47 μg poly(A)-free mRNA per 200 μl of reaction mixture. (Reproduced from Soreq, H., Nudel, U., Salomon, R., Revel, M., and Littauer, U. Z., *J. Mol. Biol.*, 88, 242, 1974. With permission.)

lation was examined. These substances are believed to inhibit the initiation step, and have selective effects on the translation of several mRNAs. The two forms of actin mRNA were affected to the same extent by either inhibitor. Actin synthesis in the wheat germ system was also found to be severely inhibited in the presence of excess mouse sarcoma mRNA, but both forms of the actin mRNA were affected in this manner.

The studies of cell-free translation have yielded ambiguous results so far. In two separate studies, the mRNA depleted of poly(A) seemed to be more susceptible to degradation, but the effects were rather small. No such effect was observed in a third study, but the latter involved ovalbumin mRNA instead of globin mRNA, and was carried out in a different cell-free system. In the same study, an effect on initiation efficiency was suggested, but only when the reticulocyte cell-free system was used. Clearly, the characteristics of the cell-free system must be taken into consideration when assessing the results of in vitro translation. In any case, cell-free systems are probably not suitable for the detection of subtle differences in mRNA translation efficiency and stability.

B. Translation in Xenopus Oocytes

It has been possible to obviate some of the shortcomings of the cell-free systems by carrying out the translation assays in intact frog eggs. Globin mRNA injected into the eggs is effective in promoting globin synthesis. Moreover, it remains functional for several days. The behavior of globin mRNA depleted of poly(A) was sharply different.[49] This material did promote some globin synthesis, but it remained active for only a few hours (Figure 10). When the fate of injected RNA preparations was followed by hybridization of extracted RNA with globin cDNA, it was found that the poly(A)-lacking globin mRNA disappears rapidly, while the untreated mRNA remains stable.[50] Functional poly(A)-containing globin mRNA could be reconstituted from the depleted chains by incubation with bacterial poly(A) polymerase and ATP.[51] This shows that the original enzymic treatment had affected only the poly(A) sequence.

It was possible to determine whether the degree of stability of the injected mRNA was dependent on the length of the poly(A) sequence. Globin mRNA was subjected to controlled phosphorolysis in order to obtain preparations in which defined portions of the poly(A) sequence were retained.[52] Preparations with sequences containing 30 or more AMP residues seemed to be as stable in the oocytes as the untreated mRNA, while those with only 16 AMP residues behaved as if they had no poly(A) at all (Figure 10). Two important concepts can be derived from this study. One is that the poly(A) sequence exerts an all or none effect on the mRNA to which it is attached, regardless of its length. The other is that there is a critical poly(A) size, below which this sequence is not functional. Some of the characteristics of mRNA behavior in intact cells tend to support at least one of these concepts (see Section IV.C.).

The studies with oocytes have also provided some insight into the process of mRNA degradation. It was possible to examine the fate of mRNA depleted of poly(A) under conditions that prevent its translation in the oocytes. Rabbit α-globin mRNA is translated very poorly in this system in the absence of hemin. The poly(A)-depleted form of this RNA species remained quite stable under these conditions.[53] It appears, therefore, that the degradation of mRNA chains that have lost their poly(A) sequence may be linked to their translation.

The study of additional mRNA species in the oocyte system has led to seemingly contradictory results. A poly(A) sequence was added enzymatically to HeLa cell histone mRNA, and this caused a marked increase in the functional stability of these RNA species, in agreement with the results obtained with globin mRNA.[54] The mRNA for interferon, however, showed about the same stability whether a poly(A) sequence was present or not.[55] The case of interferon mRNA may be unusual, since even the intact mRNA chains were relatively short lived in the oocytes. It could be that the poly(A) sequence is lost rapidly from this mRNA species after injection. This would explain why prior removal of this sequence had no effect on stability. Poly(A)-containing mRNAs can vary widely with respect to metabolic stability and it is possible that differences in the rate of poly(A) loss are related to this variability (see Section V.D.).

The studies with the oocyte system have shown clearly that the poly(A) sequence can play an important role in promoting mRNA stability. It remains to be determined whether the conditions that prevail in the oocytes are comparable to those that exist in normal cells. There is an additional uncertainty due to the fact that deproteinized RNA was used in these studies. If the mRNA must function as a nucleoprotein complex in the cells, then the injected RNA preparations would have to acquire the required protein complement, if it is available in the oocytes.

C. Behavior in Intact Cells

The possibilities for studying the effect of poly(A) on the behavior of mRNA in its

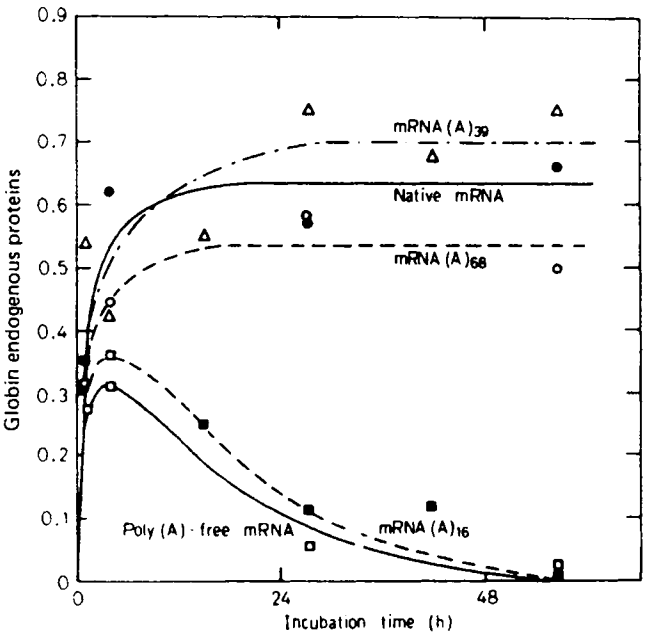


FIGURE 10. The rate of globin synthesis relative to endogenous protein synthesis in oocytes injected with native mRNA, mRNA(A)$_{68}$, mRNA(A)$_{39}$, mRNA(A)$_{16}$ and poly(A)-free mRNA. (A)$_i$ indicates length of poly(A) sequence. (Reproduced from Nudel, U., Soreg, H., Littauer, U. Z., Marbaix, G., Huez, G., Leclerq, M., Hubert, E., and Chantrenne, H., *Eur. J. Biochem.*, 64, 119, 1976. With permission.)

natural environment are rather limited. Newly synthesized mRNA chains, with a long poly(A) segment, can be distinguished from the steady-state molecules, whose poly(A) is shorter. Histone mRNA, which lacks poly(A), can be compared to other, poly(A)-containing species. Ideally, poly(A)-containing and poly(A)-lacking chains of the same mRNA species should be compared.

Studies of mRNA turnover in exponentially growing cells have shown that the poly(A)-containing species decay by first-order kinetics. This implies that the process of mRNA inactivation is random, with new and old molecules having the same probability of being destroyed.[36] Thus the size of the poly(A) sequence would seem to have no bearing on the stability of the molecule.

The stochastic nature of the poly(A)-containing mRNA decay curve has been confirmed in many cell types. Analysis of decay curves of mRNA labeled for brief periods has revealed the existence of more than one stability class within cellular mRNA populations.[56,57] Although the shape of a multicomponent decay curve may be more difficult to interpret, it is believed that each component decays by first order kinetics (Figure 11). Moreover, the decay of a defined mRNA species, the globin mRNA, also appears to proceed in a stochastic fashion.[58]

The behavior of the histone mRNAs differs from that of the poly(A)-containing species.[36] In this case, the decay curve is not indicative of a first order process. It can be accounted for by assuming that the histone mRNAs are stable during a fixed period of the cell cycle, and are next destroyed rapidly. This is in agreement with the earlier finding that histone-synthesizing polysomes and the 7 to 9S polysomal RNA thought to represent histone mRNA occur only during the S phase of the cell cycle.[34] Thus the histone mRNA behaves as a stable component during the period of DNA synthesis, and becomes unstable after completion of this process. It remains to be determined

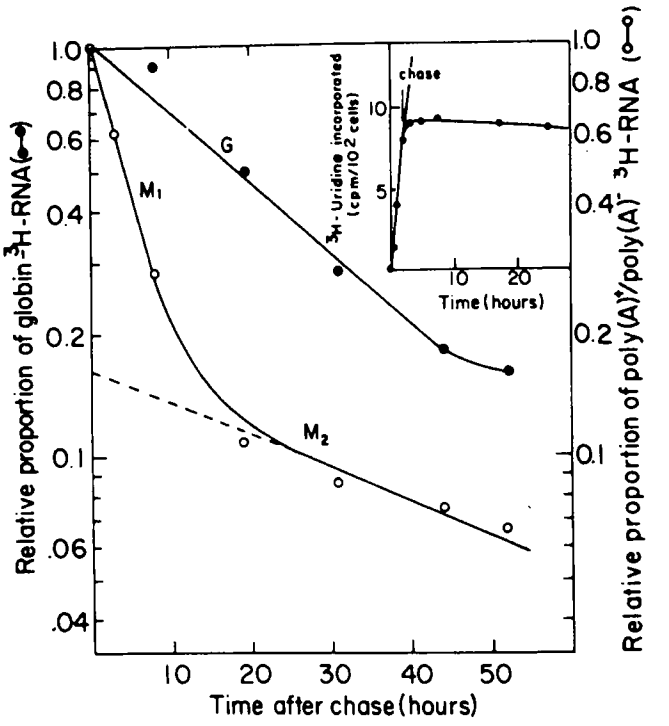


FIGURE 11. Decay kinetics of labeled globin RNA and poly(A)⁺ RNA (labeling period 2 hr). Cells seeded at 4 × 10⁴/ml were treated for 4.5 days with 1.7% DMSO with one change of medium 14 hr before labeling. The culture reached 2 × 10⁶ cells per milliliter and about 90% of the cells were benzidine-positive. Cells were labeled for 120 min and chsed. The efficiency of the chase treatment is shown in the insert. (●—●) decay kinetics of globin RNA; (○—○) decay kinetics of poly(A)⁺ RNA. (Reproduced from Aviv, H., Voloch, Z., Bastos, R., and Levy, S., *Cell*, 8, 497, 1976. With permission.)

whether this difference in behavior can be attributed to the absence of poly(A) from the histone mRNA chains.

The efficiency of mRNA translation in relation to poly(A) content has also been examined. The degree of ribosome packing on mRNA (number of ribosomes per unit length of mRNA) was taken as a measure of initiation efficiency. Rapidly labeled and long-labeled mRNA populations, which are enriched in long and short poly(A) segments, respectively, were compared with respect to distribution in polysomes of different sizes. The two populations showed similar degrees of ribosome packing, an indication that they may not differ with respect to initiation efficiency.[59] A similar approach was used to compare the poly(A)-containing and poly(A)-deficient mRNA in sea urchin polysomes.[60] The poly(A)-deficient species seemed to have a lower degree of packing in any given polysome size class. This finding would seem to indicate that mRNA species without poly(A), or with very short segments, may be less efficient in initiation. It remains to be demonstrated, however, that the pulse-labeled RNA fraction under study was in fact mRNA. Another difficulty with this approach is that the size of the noncoding regions should be taken into consideration when packing ratios are being compared. These noncoding regions can be quite considerable, and may vary widely in different mRNA species (see Section II.A.).

The unusual poly(A) content of the mRNA for β-actin has permitted the comparison

of mRNA chains with different poly(A) sizes within the same species. A substantial portion of the actin mRNA has very short segments or no poly(A) at all (see Section III.C.). The poly(A)-containing and poly(A)-deficient chains, separated by oligo(dT)-cellulose fractionation, can be compared. In this case any difference could be attributed directly to the poly(A) sequence, since the rest of the RNA chain should be the same in the two fractions. The poly(A)-deficient chains were found to be concentrated on polysomes smaller than those associated with poly(A)-containing actin mRNA.[24] The stability of the two actin mRNA classes was also compared, by following their decay in mouse sarcoma ascites cells incubated outside the animal.[45] The actin mRNA becomes unstable under these conditions. Both classes seemed to be affected to the same extent, as determined by the loss of template activity for actin synthesis. It is not sure, however, that the two types of actin mRNA could be considered as separate metabolic pools. The decay in that experiment proceeded slowly, and there could have been considerable interconversion between the two classes through poly(A) loss or elongation.

The various studies of mRNA function indicate that the size of the poly(A) sequence may be immaterial, provided that it is above a certain critical length. Messenger RNA chains with long and short poly(A) segments appear to have the same degree of stability, as indicated by kinetic studies with intact cells and by studies of globin mRNA injected into oocytes. They also appear to be equal with respect to initiation efficiency. Some differences in behavior are apparent when mRNA chains with very short segments or with no poly(A) at all are examined. These seem to be less efficient in translation, as suggested by the behavior of ovalbumin mRNA depleted of poly(A) in the reticulocyte lysate, by the distribution of poly(A)-deficient actin mRNA on polysomes of different sizes in mouse ascites cells, and by the degree of ribosome packing on the messenger-like RNA of sea urchin embryos. The poly(A)-lacking mRNA chains are also unstable when injected into oocytes, and seem more sensitive to degradation in some cell-free systems. The situation in intact cells is more confusing in this respect. The histone mRNAs can be stable or unstable, depending on the physiological state of the cell.

V. POLY(A) BREAKDOWN IN THE CYTOPLASM

A. Shortening Process

The poly(A) sequence is subject to degradation in the cytoplasm. As a result, the newly synthesized poly(A) becomes progressively shorter. The significance of this process has remained elusive, in spite of much work and speculation. At first glance, it would seem to represent a cellular mechanism for altering gradually the properties of the mRNA chains. The available evidence, however, fails to support a role for poly(A) chain length in determining the behavior of the mRNA (see Section IV.C.). A progressive erosion of the poly(A) would eventually lead to molecules which lack this sequence. The latter appear to be less effective in translation and, perhaps more importantly, seem metabolically unstable. Thus the shortening process could be part of the mechanism that leads to mRNA inactivation. However, the studies of mRNA turnover indicate that the decay of poly(A)-containing mRNA is a random process, with new and old molecules equally susceptible to degradation. This would not be consistent with a time-dependent poly(A) loss as a prerequisite for mRNA inactivation. It could be that this shortening does not represent a special cellular process designed to affect mRNA behavior. For instance, the poly(A) sequence might be exposed to the action of any 3′-exonuclease that may be present in the cytoplasm. The resulting poly(A) shortening could be unrelated to the process of mRNA inactivation.

In view of the above considerations, it is important to determine the nature of the agent responsible for the breakdown of poly(A). Clearly, this agent affects the poly(A) portion of mRNA selectively, since it leads to intact mRNA chains with shorter segments. A nonspecific endonuclease could affect the mRNA in this fashion only if the poly(A) sequence were particularly exposed. Yet the latter is known to be covered with proteins and shielded from the action of nucleases (see Section II.F.). Thus the hydrolytic agent would have to be either an endonuclease with special affinity for poly(A) or a nonspecific 3′-exonuclease.

The process of poly(A) size reduction can be followed by polyacrylamide gel electrophoresis of poly(A) segments isolated from cells exposed to radioactive adenosine. After a 5- to 10-min labeling period, the cytoplasmic poly(A) population of mammalian cells shows a distribution of sizes as narrow as that of the nuclear poly(A), but it has a slightly higher mobility.[13,16] Thus a significant reduction in size has already taken place after a 5-min stay in the cytoplasm (see Figure 3). The shortening process appears to be most rapid during the first 2 to 3 hr after entry of the mRNA in the cytoplasm. During this time the poly(A) size distribution shows relatively little spread. Beyond this initial period, the poly(A) segments become heterogeneous with respect to size, due to increase in the amounts of small segments (Figure 12). A similar pattern of change has been observed for the poly(A) sequence of mouse globin mRNA.[61]

The accumulation of small poly(A) segments has been considered as indication that poly(A) breakdown proceeds via random endonucleolytic cleavage.[14] However, it is difficult to see how such a process could lead to the gradual decrease in size evident during the first few hours. The overall changes in size distribution could be accounted for by the action of an exonuclease that attacks relatively rapidly the long, newly synthesized sequence, and that is less effective against the shorter sequences. Such heterogeneity in the time course of poly(A) breakdown could be caused by the proteins associated with this sequence. A clearer understanding of this process will require the identification of the hydrolytic enzyme and a study of its action on isolated polysomes.

B. Elongation and Turnover

The poly(A) sequence is assembled rapidly on the nuclear RNA chains. Periods of labeling as short as 1 min are sufficient to yield a homogeneous population of full-length segments.[61] In addition to this rapid *de novo* synthesis, there occurs a slow elongation of the completed nuclear segments. This is indicated by the fact that brief periods of ^{3}H-adenosine incorporation cause mostly terminal labeling of preexisting poly(A) sequences.[13,62] The end-addition process can be distinguished from *de novo* synthesis by its insensitivity to 3′-deoxyadenosine, a drug that inhibits poly(A) synthesis.[63]

Poly(A) elongation occurs in the cytoplasm as well as in the nucleus. The terminal labeling of the steady-state population of poly(A) segments can be detected in cells labeled for brief periods. In addition to the newly synthesized component of large size, a broad peak is seen in polyacrylamide gel profiles of labeled poly(A) (Figure 13). The latter corresponds to the steady-state poly(A) and consists of chains with 2 to 6 labeled residues at the 3′ end.[13,62] The elongation process can be followed for longer periods in cells pretreated with actinomycin D, since influx of heavily labeled, newly synthesized poly(A) from the nucleus is prevented.[63] In these cells, the labeling of cytoplasmic poly(A) reaches a plateau within about 10 min, while it continues in a linear fashion in the nucleus (Figure 14). The situation in the cytoplasm can be accounted for by the concomitant poly(A) degradation process which seems to gnaw at the 3′ end of the poly(A) sequences. There is no evidence for a poly(A) shortening process in the nucleus, and the terminal addition in this cell compartment can result in the generation

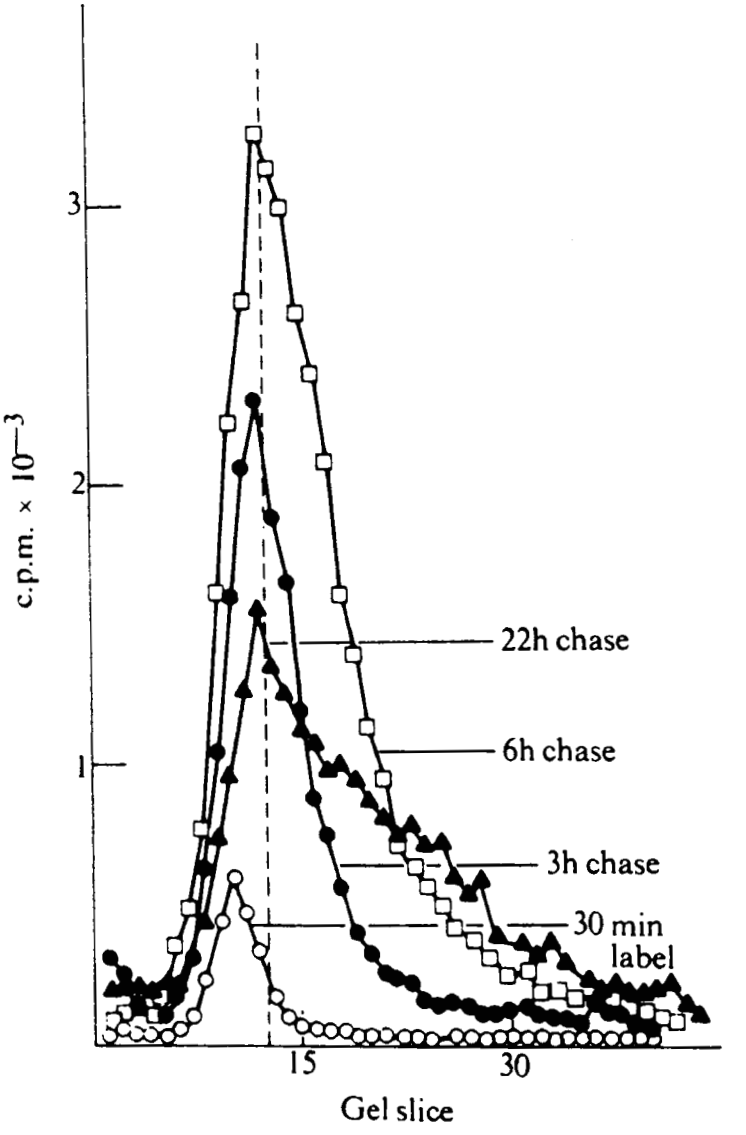


FIGURE 12. Effect of cell growth in unlabeled medium on electrophoretic migration of labeled poly(A). Cells were labeled for 40 min with 5 mCi of ^{3}H-adenosine. A sample was removed at this time and the remaining cells were collected by centrifugation and resuspended in fresh medium at 2×10^6 cells per milliliter. One third of the culture volume was removed after 3, 6, and 22 hr and polysomal RNA prepared. The polysomal RNA was digested, and samples were phenol-extracted and collected by precipitation with ethanol, dissolved, and passed through a column of poly(U)-sepharose. This procedure was necessary to rid the samples of fragments originating from labeled rRNA so that the presence of small pieces of poly(A) could be observed. The column eluates were precipitated and subjected to electrophoresis on 15% polyacrylamide gels containing 8 *M* urea which insures no aggregation after the column procedure. (Reproduced from Scheiness, D. and Darnell, J. E., *Nature (London) New Biol.*, 241, 266, 1973. With permission.)

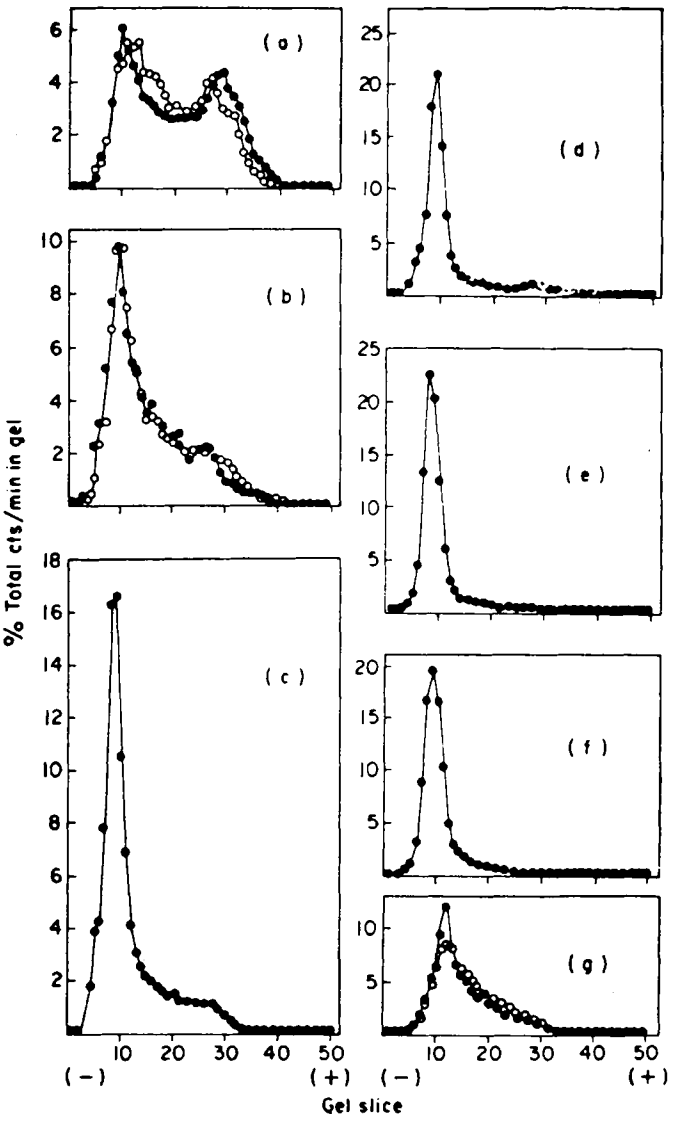


FIGURE 13. Growing HeLa cells were concentrated to 3×10^{-6} cells per milliliter and labeled with ^{3}H-adenosine (50 to 125 μCi/ml) for periods of 40 min or less; cells were grown for 3 days in ^{3}H-adenine (5 to 10 μCi/ml, 50 μM-adenine). The poly(A) from the extramitochondrial cytoplasmic fraction was examined by electrophoresis. Data for two experiments are given for the 1- and 2-min time periods. The results are expressed as the percent of cpm in each gel slice relative to the total number of cpm on the gel. (a) 1 min; (b) 2 min; (c) 5 min; (d) 10 min; (e) 20 min; (f) 40 min; (g) 3 days. (Reproduced from Sowicki, S. G., Jelinek, W., and Darnell, J. E., *J. Mol. Biol.*, 113, 219, 1977. With permission.)

of abnormally long sequences. This is observed in cells treated with actinomycin D,[63] and in cells subjected to starvation.[64] A net lengthening of poly(A) due to elongation in the cytoplasm has been observed in fertilized sea urchin embryos.[65]

The significance of the terminal addition in the cytoplasm of mammalian cells is not clear.. It could counterbalance the process of poly(A) degradation, but it is not known whether it can affect significantly the steady-state size of the poly(A) sequence. At-

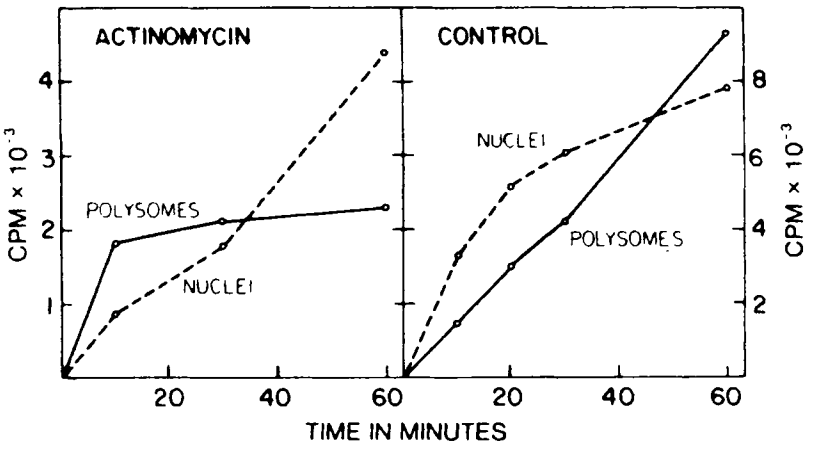


FIGURE 14. Effect of pretreatment with actinomycin on time course of poly(A) labeling in Chinese hamster cells. Cell suspensions were incubated in the presence or absence of actinomycin for 30 min before addition of labeled adenosine. Data from untreated cells and actinomycin-treated cells are from separate experiments, and the absolute levels of incorporation into poly(A) are not comparable. (Reproduced from Diez, J. and Brawerman, G., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4091, 1974.)

tempts have been made to determine whether the entire poly(A)-containing mRNA population is subject to the elongation process. It is possible to estimate the number of chains being elongated by measuring the radioactivity in the 3′-terminal nucleotide, which is released as adenosine upon alkaline hydrolysis. One such study, in which the numbers of steady-state and newly labeled termini were compared, led to the conclusion that 30 to 50% of the mRNA chains were being elongated at any one time.[61] Another study involved the comparison of elongating termini in nucleus and cytoplasm. This led to a lower estimate of the cytoplasmic chains being subject to end-addition, but the contribution of short segments unable to bind to Millipore® filters was missed in the latter study.[13] A comparison of labeled termini on poly(A) segments of different lengths indicated that all size classes may be equally susceptible to elongation.[61]

Is there a relation between the poly(A) chain extension process in the cytoplasm and mRNA function? A crude estimate of the rate of end-addition relative to influx of new poly(A) into the cytoplasm can be obtained by comparing the levels of internal and terminal labeling at different time intervals.[13] Using this criterion, no obvious differences in elongation were seen when protein synthesis was inhibited by exposure of the cells to cycloheximide (Table 3). Also, the poly(A) sequences in free mRNP particles and in active polysomes showed the same pattern of labeling. Thus the poly(A) chain-extension process seems to be unrelated to translation. Significant differences in the relative rates of cytoplasmic poly(A) elongation were detected when mouse sarcoma 180 ascites cells were compared to Chinese hamster cells,[15] but the nature of the relevant physiological parameter is not known.

C. Relation of Poly(A) Decay to Cell Metabolism

In order to gain information on the physiological significance of the poly(A) shortening process, attempts have been made to correlate it with mRNA function. For instance, a linkage between rate of shortening and translation could signify that the sequence somehow controls the functional lifetime of the mRNA molecule. The concept of a "ticketing" process that defines the number of rounds of translation that an

Table 3
RELATIVE AMOUNTS OF LABELING IN INTERNAL AND 3′ TERMINAL AMP RESIDUES OF POLY(A) PREPARATIONS FROM MOUSE ASCITES CELLS TREATED WITH CYCLOHEXIMIDE[13]

	Control		Cycloheximide	
Cell fraction	10 min	30 min	10 min	30 min
Nuclear	132	199	125	179
Cytoplasmic	29	85	34	89

Note: Mouse sarcoma cells were incubated for 30 min in presence or absence of cycloheximide (100 μg/mℓ), and incubations continued after addition of ^{3}H-adenosine. Samples taken at specified time intervals after exposure to label. Poly(A) preparations from cell samples were subjected to alkaline hydrolysis and amounts of radioactivity in AMP and adenosine determined. Values expressed as ratios of labeled AMP/adenosine.

mRNA chain can accomplish has been advanced.[66] Sequential removal of adenylate residues somehow linked to the translation initiation process seemed like a possible way to achieve such a ticketing.

The effect of treatments that inhibit protein synthesis on the poly(A)-shortening process was examined by comparing the size distribution of labeled poly(A) segments at different time intervals after exposure of the cells to radioactive adenosine. Cycloheximide, an inhibitor of polypeptide chain elongation, had no effect on the extensive shortening that takes place during the first hour in mouse sarcoma cells.[64] Emetine, another such inhibitor, was first reported not to have any effect on poly(A) shortening in HeLa cells.[16] A subsequent report did show some effect in the treated cells (see below). Neither inhibitor affected the decrease in poly(A) size in mouse globin mRNA.[62]

In a different approach to the study of poly(A) shortening in relation to translation, the sequences of free mRNP particles and of mRNA engaged in translation have been compared. Mouse sarcoma polysomes and particles showed similar changes in poly(A) size distribution.[64] In sea urchin embryos, however, the poly(A) sequences of free mRNPs were shown to decrease in size more slowly than those of polysomal mRNA.[67] It is not impossible that different types of mRNP particles would behave differently in this respect. For instance, the mammalian particles in the above study may have been in equilibrium with polysomal mRNA, while the sea urchin particles could represent a special class of mRNA maintained in the inactive state. The possibility remains, therefore, that the poly(A) sequence in untranslated mRNA is less subject to the decay process.

The behavior of the poly(A) of free RNP particles has also been studied in mouse sarcoma cells subjected to starvation. This treatment causes a block in polypeptide chain initiation, and results in the release of polysomal mRNA as RNP particles. The poly(A) sequence showed a decrease in size, but to a lesser extent than in cells incubated in full medium.[64] It is not sure, however, that the starvation conditions affected the rate of poly(A) breakdown. The overall size of the nuclear poly(A) segments increased considerably in the starved cells. If RNA chains with the larger poly(A) sequences were

being transferred to the cytoplasm, then the normal rate of decay could have resulted in larger sizes for the poly(A) of the mRNP particles after a given time interval.

An effect of emetine on the poly(A) size distribution in HeLa cells has been reported.[14] The size-reduction process seemed to proceed more slowly in the treated cells. This would seem to contradict the results of the other studies, but it is not certain that the effect on poly(A) shortening was related to the block in protein synthesis. The prolonged treatment with emetine could have affected the cells in other ways. It was observed that incubation of mouse sarcoma ascites cells for more than 2 hr in a culture medium tends to inhibit the poly(A) shortening process.[64] Exposure of mouse myeloma cells to excess adenosine also affected this process.[91] The poly(A) shortening has been observed to proceed more slowly in cultured mammalian cells growing under less-than-optimal conditions.[92] Thus, it appears that the process of poly(A) shortening can be affected under certain conditions, but there does not seem to be any direct link between this process and mRNA translation.

D. Relation to mRNA Stability

Individual mRNA species show defined rates of decay that can vary within a wide range. Moreover, the stability of a given mRNA species can be altered under certain conditions.[68] Since poly(A) seems to be implicated in promoting mRNA stability, the possibility has been considered that the rate of decay of this sequence determines the lifetime of the mRNA chain to which it is attached. The rate of loss of the poly(A) could itself be controlled by the proteins associated with this sequence, and by the cytoplasmic elongation process.

There is relatively little information available on the susceptibility of individual mRNA species to poly(A) shortening. The initial stages of this process seem to affect the bulk of the mRNA to a similar extent, since the size distribution of the shortening poly(A) segments remains quite narrow for the first 1 to 2 hr. Some mRNA species, such as the RNA for actin, have a distinct steady-state poly(A) size distribution. This could be the result of a more rapid rate of poly(A) size decrease, although other explanations are possible.

Direct evidence for differential poly(A) stability comes from the study of two adenovirus mRNA species in transformed rat cells.[69] These two species differ considerably with respect to metabolic stability, and both are far less stable than the bulk of the cellular mRNA. The poly(A) size distribution was measured by stepwise elution of pulse-labeled RNA from poly(U)-sepharose. Distributions of the specific mRNA species in different fractions were determined by annealing to DNA probes. When the fractionation was applied to preparations from cells labeled for different time periods, it was observed that the more unstable of the two mRNA species shifted more rapidly toward a population with short poly(A) segments (Figure 5). The cellular mRNA showed a much slower poly(A) size decrease. This study shows that the poly(A) sequence of individual mRNA species can be degraded at different rates. It also indicates that there may be some link between the rate of decay of an mRNA species and the rate of degradation of its poly(A) sequence. Such a relation was also suggested by the fact that exposure of cells to emetine slows down both the poly(A) shortening and the decay of cellular mRNA. In the latter case, however, the two effects could have been independent consequences of the emetine treatment (see Section V.C.).

The above results could be interpreted by assuming that an exonuclease works its way gradually through the poly(A) sequence, and that the complete removal of this sequence leaves the functional portion of the mRNA vulnerable to an inactivating process. Such a mechanism, however, would be incompatible with the observed stochastic decay of mRNA. This difficulty could be overcome by having an endonuclease

as the poly(A)-degrading enzyme. This enzyme would attack the poly(A) in a random fashion, and hits close to the body of the mRNA would render it susceptible to inactivation.[69] Such a process would result in stochastic mRNA decay. However, the initial rapid stage of poly(A) shortening does not appear to be due to random endonucleolytic cleavage (see Section V.A.). Also, the offending endonuclease would have to be highly specific for poly(A), in order to account for the selective degradation of this sequence in the functioning mRNA.

The manner in which the poly(A) sequence evolves in the adenovirus mRNAs suggests that the steady-state size distribution is reached rapidly in the short-lived species (Figure 5). An exonucleolytic process that is very rapid at first, and is slowed down considerably when a certain poly(A) size is reached, could perhaps account for the observed behavior. The linkage between poly(A) degradation and mRNA decay could be explained by having the latter process operate at the level of the steady-state molecules. Such a scheme would result in stochastic mRNA decay only after the steady-state size distribution has been reached. This kind of deviation from exponential decay would probably be difficult to detect, particularly in the case of a complex population of mRNA species with many different half-lives. It should be noticeable in the decay curve of an individual mRNA species. It is difficult, however, to make precise measurements of this kind. Moreover, experimental points that do not fit the simple exponential decay curve tend to be overlooked.

VI. POLY(A) AND mRNA BIOGENESIS

A. Relation of Poly(A) Addition to mRNA Processing

Poly(A) addition is part of the complex process by which primary transcripts are converted to functional mRNA. The possible involvement of the poly(A) segment in some of the nuclear events that lead to mRNA production has been the subject of much investigation. One approach to this problem has been to determine the timing of poly(A) addition in relation to other processing steps. The early studies dealing with the polyadenylylation step have involved kinetic measurements of overall poly(A) labeling in the nucleus, and size measurements of the nuclear molecules being subjected to poly(A) addition. These have led to inconclusive results. With the advent of specific DNA probes, it has become possible to identify the nuclear precursors of individual mRNA species, and to define some of the processing steps.

B. Poly(A)-Containing mRNA Precursors

A 15S nuclear precursor to β-globin mRNA has been identified. It contains, in addition to the nucleotide sequences of the mRNA, two intervening sequences that are also known to be present in the corresponding structural gene.[70] The poly(A) sequence is also present in this molecule.[70,70a] This finding would seem to indicate that poly(A) addition precedes the excision of the intervening sequences from the transcript. The 15S β-globin RNA chain seems to be the largest well-defined precursor identified so far. The occurrence of a 27S RNA chain that contains β-globin mRNA sequences has been reported,[58] but most investigators have not been able to confirm this finding. According to the available evidence, therefore, the 15S RNA would seem to represent the primary transcript, and poly(A) addition would appear to be an early step in mRNA processing. In an effort to determine whether all 15S globin RNA chains carry a poly(A) sequence, the amounts of this material in total nuclear RNA and in RNA bound to oligo(dT)-cellulose have been compared. At least 50% of the 15S component was recovered in the poly(A)-containing fraction.[71,72] A more direct measurement that involves the comparison of adsorbed and unadsorbed RNA fractions indicated that at

most, 10 to 20% of the 15S RNA may lack poly(A).[73]

A study of the nuclear precursors to mouse immunoglobulin mRNAs has yielded similar results.[74] RNA transcripts of 11 and 5.3 kilobases, that bear the polynucleotide sequences of the heavy- and light-chain mRNAs, were detected. Several smaller-sized chains, presumably processing intermediates, were also present. All species, including the largest transcripts detected, were found to carry a poly(A) sequence. The majority of the light-chain precursor RNA molecules were recovered in the RNA fraction adsorbed on oligo(dT)-cellulose. In the case of the heavy-chain precursor RNAs, the yields were about 50%. The amounts of precursors in the poly(A)-lacking fraction were not measured.

Studies of adenovirus transcription products in infected cells have also yielded significant information on the relation of poly(A) addition to mRNA processing. A 28S precursor to the 20S mRNA for the adenovirus-2 DNA-binding protein was found to be present in the poly(A)-containing nuclear RNA fraction.[75] This mRNA molecule contains two intervening sequences, and is the largest precursor detected for this mRNA species. If one assumes that the 28S RNA chain represents the primary transcript, then poly(A) addition would represent the first step in the processing of this RNA species (Figure 15). A 23S intermediate, which presumably has undergone one of the two required splicing events, was also identified in the polyadenylylated fraction. It was not stated whether this species occurred exclusively as a poly(A)-containing molecule. Without this information, it is not possible to conclude that the presence of the poly(A) sequence is essential for the first splicing event.

A study of the late SV-40 transcript in infected cells has provided evidence for a processing step that precedes poly(A) addition.[76] The site of polyadenylylation for this transcript was localized at position 0.17 of the circular SV-40 map. Larger transcripts lacking poly(A) were identified, and the positions of their 3′ termini were found to be located in a broad region of the genome extending beyond position 0.28. This suggests that transcription proceeds past the poly(A) addition site, and that the primary transcript must undergo a highly specific cleavage in order to yield the 3′ terminus for polyadenylylation.

C. Poly(A) Addition and mRNA Selection in Adenovirus-Infected Cells

The study of the large late adenovirus-2 transcription unit has provided considerable insight into the process of poly(A) addition. This region of the viral genome yields 13 to 14 different mRNA species, grouped in five 3′ coterminal classes (see Figure 16). Therefore, five different polyadenylylation sites occur in this transcription unit. It has been shown that the RNA polymerase transcribes the entire region as a continuous RNA chain, and that transcription terminates beyond the last site for poly(A) addition.[78] Thus the 3′ terminus of each of the mRNAs encoded in the transcription unit is generated by an internal cleavage of the primary transcript, followed by, or concurrent with, addition of poly(A).[77]

The generation of the 3′ terminus for polyadenylylation appears to play a major role in the selection of the adenovirus mRNA species to be expressed. In principle, only one of the 13 to 14 species can be generated from a primary transcript, because each must be spliced to the leader sequences, which appear to be represented only once in the transcription unit (see Figure 16). Labeling studies have shown that the RNA sequences adjacent to the newly generated poly(A) are transferred in a nearly quantitative fashion to the cytoplasm as part of mRNA.[77] It appears, therefore, that only the site that contains the 3′ end of the mRNA species to be expressed receives a poly(A) sequence.

Several splicing events are required to produce a mature mRNA species from the

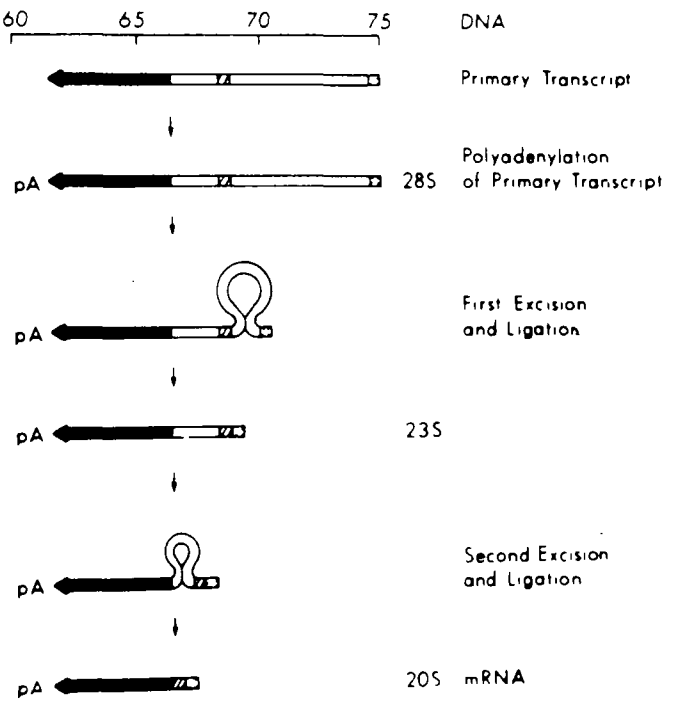


FIGURE 15. Proposed steps in the maturation of mRNA transcribed from early region 2 of adenovirus DNA. (Reproduced from Goldenberg, C. J. and Raskas, H. J., *Cell*, 16, 135, 1979. With permission.)

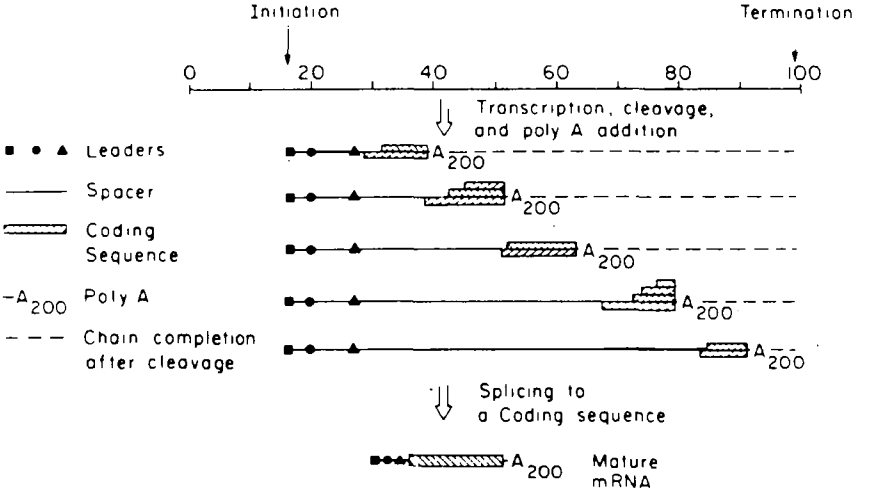


FIGURE 16. Schematic diagram depicting the proposed sequence of events in the processing of a primary transcript from the late adenovirus *16-99* transcription unit. (Reproduced from Nevins, J. R. and Darnell, J. E., *Cell*, 15, 1490, 1978. With permission.)

late adenovirus transcript. First the leader sequence must be assembled from three separate segments, through removal of two intervening sequences. The leader then must be spliced to the main body of the mRNA. The latter step involves the selection of the mRNA species to be expressed within a 3′ coterminal group. The timing of

poly(A) addition in relation to the splicing steps was studied by following the appearance of labeled poly(A)-containing RNA chains.[77] These were generated within 1 min of their transcription, and were larger than the corresponding mRNAs. This indicates that polyadenylylation precedes the final splicing step. The study led also to the conclusion that poly(A) addition may precede all splicing events, but the size measurements were probably not sufficiently precise to exclude the possibility of some splicing in the leader sequence having occurred prior to polyadenylylation.

A different approach has yielded additional information on the relation of splicing to poly(A) addition in the late adenovirus transcript. In this study, adenovirus-specific RNA, synthesized in isolated nuclei, was analyzed, as well as the RNA produced in vivo.[79] Comparison of the 5′-terminal regions of processing intermediates present in poly(A)-containing and poly(A)-lacking RNA fractions led to the conclusion that the initial splicing which generates the leader sequence may be independent of the poly(A) addition step, and may possibly take place on nascent chains. The final splicing event, however, seemed to take place exclusively on polyadenylylated molecules. It was also observed in that study that some of the 3′ coterminal sites seem to be polyadenylylated more efficiently than others. These correspond to the most abundant mRNA species found in the cytoplasm. This finding led to the suggestion that the poly(A) addition step may influence the degree of abundance of a given mRNA species.

Several important features of the poly(A) addition process have emerged from the study of viral mRNA processing. It occurs on a 3′ terminus generated within the primary transcript, and this internal cleavage followed by polyadenylylation marks the 3′ end of the future mRNA molecule. Also the splicing reaction that joins the 3′ terminal segment to the rest of the mRNA occurs on the polyadenylylated chain. Since the major late adenovirus transcript is polycistronic, and since only one mRNA species can be generated from each transcript, the processing steps must be highly selective. Of the five potential 3′ terminal sites, only one undergoes cleavage plus poly(A) addition. Moreover, the splicing to the leader sequence occurs within the 3′ coterminal group that has received the poly(A) sequence. These characteristics indicate that polyadenylylation may play an important role in the selection of the RNA sequences destined to become the mRNA.

The processing steps for the formation of cellular mRNA are less well-defined. Several precursors for individual mRNA species have been identified (see Section V.B.), but it is not sure that any of them represents a primary transcript. Thus it is not known whether poly(A) addition takes place at the terminus of transcription or on a site generated by internal cleavage. The occurrence of poly(A) on these larger precursors would tend to indicate that the addition of this sequence must precede at least some of the splicing steps. It cannot be excluded, however, that splicing and poly(A) addition take place independently of each other, since a portion of these precursors and intermediates appears to be present in the poly(A)-lacking fraction.

D. Conservation of Nuclear Poly(A)

If poly(A) addition were in fact to play a major role in the selection of cellular mRNA sequences among the nuclear transcripts, then the majority of the poly(A) produced in the nucleus should end up in the cytoplasm as part of the mRNA. Studies of the kinetics of labeling have been carried out to determine the degree of nuclear poly(A) conservation, but results of these studies have not lent themselves to easy interpretation. For instance, a careful study of the time course of labeling of nuclear poly(A) showed that this sequence accumulates in the nucleus for several hours.[80] If all the newly synthesized nuclear poly(A) were to function as precursor to cytoplasmic poly(A), then the rate of appearance of the latter should have kept increasing during

this time period. However, the amount of labeled poly(A) started to increase in the cytoplasm at a linear rate very soon after exposure of the cells to radioactive adenosine. This led to the conclusion that much of the nuclear poly(A) does not become part of cytoplasmic mRNA. It was found subsequently that a large portion of the mRNA belongs to an unstable class with a t½ of about 1 hr.[57] Thus part of the emerging poly(A) is lost rapidly, making it difficult to measure the rate of appearance of this sequence in the cytoplasm. A significant amount of cytoplasmic poly(A) label must also be lost through the rather rapid shortening of this sequence (see Section V.A.).

Another complicating factor in the interpretation of labeling kinetics is the elongation of preexisting poly(A) that takes place in the cytoplasm (see Section V.B.). This process is likely to affect significantly the early stages of the cytoplasmic labeling curve thus making it appear that newly synthesized poly(A) enters into the cytoplasm without any lag. This apparent rapid entry of newly synthesized poly(A) into the cytoplasm[8,80] had led to the conclusion that poly(A) addition in the nucleus is a late event in mRNA biogenesis.

In the study of adenovirus transcripts discussed in Section VI.C., the degree of conservation of sequences adjacent to poly(A) was determined by following the labeling of poly(A)-containing RNA chains in nucleus and cytoplasm, using uridine as the labeled precursor.[77] This approach avoids the problems due to the complexity of poly(A) metabolism. Also the degree of conservation of precursors to individual mRNA species could be determined. For some of these species, the transfer of polyadenylylated segments from nucleus to cytoplasm seemed nearly quantitative.

Substantial evidence for a less than quantitative transfer of poly(A)-terminated RNA sequences from nucleus to cytoplasm has come from a comparison of resting and growing mouse fibroblasts.[81] The growing cells have 2 to 4 times as much poly(A)-containing RNA in their cytoplasm. This difference does not appear to be due to a higher rate of transcription in the growing cells, but rather to more efficient processing of the nuclear transcripts. The fraction of the nuclear RNA that is polyadenylylated is also the same in the two types of cell cultures. Comparison of labeling kinetics in resting and growing cells indicated that the latter transfer a greater faction of their nuclear poly(A) into the cytoplasm. While this study did not provide information on the degree of conservation of poly(A)-terminated nuclear RNA chains, it showed that the extent of transfer of these chains to the cytoplasm can vary. This finding raises the possibility that cellular mRNA levels may be controlled in part by the extent to which poly(A)-containing nuclear RNA chains are conserved for transfer to the cytoplasm.

E. Comparison of Nuclear and Cytoplasmic RNA Sequences Adjacent to Poly(A)

The studies of labeling kinetics have provided some information on the extent to which RNA segments selected for polyadenylylation are preserved for export to the cytoplasm. They have not indicated whether only those sequences destined to become part of the cytoplasmic mRNA receive a poly(A) sequence. It could be that acquisition of this sequence is what renders an RNA chain competent for further processing, and causes its preservation in the nucleus until the maturation process is completed. It is also possible, however, that polyadenylylated sequences to be exported are selected among a larger pool of poly(A)-containing RNA species.

The question whether poly(A) addition is the decisive selection step has been studied through the comparison of nuclear and cytoplasmic RNA sequences adjacent to poly(A). The nuclear RNA of HeLa cells was used to prepare DNA complementary (cDNA) to the poly(A)-containing sequences.[82] About 50% of this cDNA could anneal to cytoplasmic poly(A)-containing RNA (Figure 17); but, since the homologous reac-

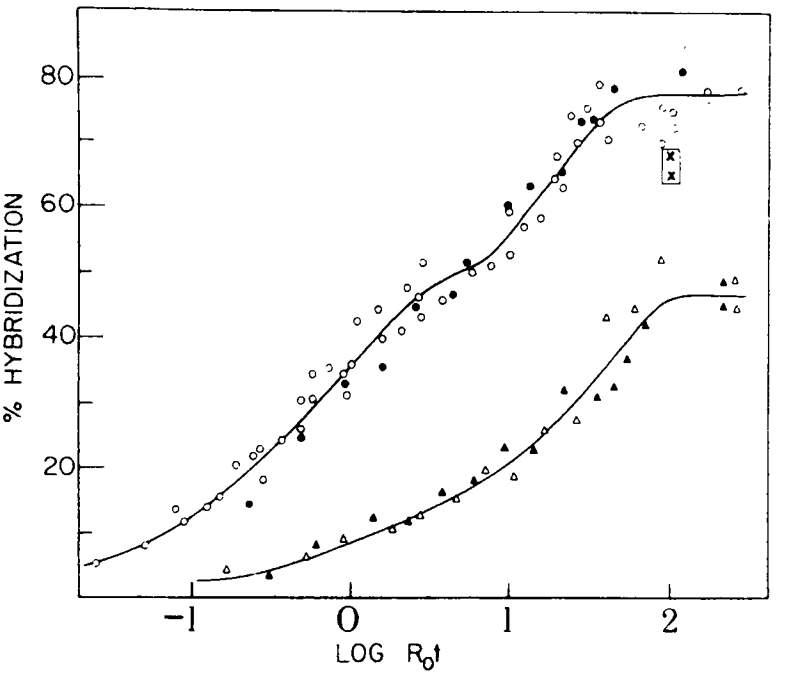


FIGURE 17. Hybridization of cDNA transcribed from poly(A)⁺ hnRNA fragments. Poly(A)⁺ nuclear RNA fragments and poly(A)⁺ mRNA were purified and cDNA was prepared from each. RNA excess hybridizations were performed using a 1000- to 2000-fold excess of driver RNA. mRNA concentrations were calculated from the poly(A) content of the preparation assuming that the poly(A) is 4% of the chain length. (△, ▲) nuclear cDNA driven by poly(A)⁺ mRNA (×) nuclear cDNA driven by poly(A)-containing hnRNA fragments, (○, •) message cDNA driven by poly(A)⁺ mRNA. Different symbols indicate different RNA preparations used to prepare cDNA. (Reproduced from Herman, R. C., Williams, J. G., and Penman, S., *Cell*, 7, 437, 1976. With permission.)

tion was less than quantitative, the degree of homology was probably close to 70%. The nuclear RNA chains used for cDNA preparation had been trimmed to a size comparable to that of the cytoplasmic RNA, in order to avoid copying nuclear sequences that are excised during processing. A similar study of mouse brain transcripts also showed that most of the nuclear sequences adjacent to poly(A) are represented in the cytoplasm.[83]

The portion of the cDNA that could not anneal to cytoplasmic mRNA was considered to represent a special class of polyadenylylated species that are not meant to be converted to mRNA.[82] It was not shown, however, that these sequences belong to separate poly(A)-containing chains. They could represent intervening sequences of genuine mRNA precursors, that are excised during processing.

Studies of RNA transcripts from mouse erythroleukemia cells have led to drastically different results. The sequence complexities of the poly(A)-containing nuclear and cytoplasmic RNA fractions was compared by measuring the kinetics of hybridization to their respective cDNAs. The nuclear sequences showed a fivefold higher degree of complexity.[84] In a separate study, nuclear and cytoplasmic poly(A)-containing RNA preparations were annealed to highly radioactive mouse DNA, in order to determine what portion of the genome is represented in each fraction. The nuclear polyadenylylated RNA was complementary to 3 to 8% of the haploid genome, while its cytoplasmic counterpart contained only 1 to 2% of the genomic sequences.[85] These results tend to

indicate that the majority of the nuclear RNA species that receive a poly(A) segment are not converted to mRNA. Moreover, the sequence complexity of the total nuclear RNA of the erythroleukemia cells seemed nearly equal to that of its poly(A)-containing component.[85] This would imply that all nuclear transcripts and processing products become polyadenylylated. However, the approach used for the study of erythroleukemia cell transcripts is subject to uncertainties. The nuclear RNA segments used in this study were not trimmed to a standard size prior to poly(A) selection, and the longer nuclear RNA precursors could have contributed to the excess sequences found in the nuclear molecules. Moreover, the results could have been affected by failure to prepare a poly(A)-containing nuclear RNA fraction free of contamination by chains lacking poly(A). In the approach used for the study of HeLa cell and brain transcripts, the results did not depend on efficient separation of poly(A)-containing and poly(A)-lacking nuclear RNA chains, since only the nuclear cDNA primed by oligo(dT) was used for the study of nuclear sequences.

F. Inhibition of Poly(A) Synthesis by 3′-Deoxyadenosine

An early indication that the addition of a poly(A) segment to nuclear RNA chains may be essential for mRNA processing had been provided by the observation that cordycepin, a 3′-deoxy analog of adenosine, blocks the appearance of labeled mRNA into the cytoplasm.[86] Heterogeneous nuclear RNA synthesis was relatively unaffected by this treatment, an indication that the drug may not have interfered with transcription. It was found subsequently that poly(A) synthesis is inhibited preferentially by cordycepin.[8,87] This finding led to the suggestion that the poly(A) sequence may be required for the transport of the mRNA molecule from nucleus to cytoplasm. Such a role in transport has not been substantiated, but the cordycepin effect suggests that poly(A) addition may play an essential role in mRNA production. However, the assumption that normal transcription takes place in the presence of the drug has not been verified.

The effect of cordycepin on the expression of the globin genes in mouse erythroleukemia cells has been examined, using globin cDNA as a probe for the detection of globin-specific transcripts.[88] The production of the β-globin mRNA precursor was found to be severely depressed in the treated cells. This led to the conclusion that transcription of the globin gene is inhibited in cells treated with cordycepin. It is also possible to interpret the data in terms of a rapid destruction of the primary transcript.

Results obtained with the use of a metabolic inhibitor are usually difficult to interpret, but it is possible that a detailed analysis of transcription products in cells exposed to cordycepin would provide significant insight into the mechanism of mRNA processing.

CONCLUSION

The bulk of the evidence discussed in this review points towards two major roles for the poly(A) sequence. It seems to participate in the selection of the 3′-terminal regions of the mRNA chains during their biogenesis in the nucleus, and it appears also to be part of the process that controls mRNA stability in the cytoplasm.

The biochemical mechanisms by which these functions may be achieved are still a matter of conjecture. The occurrence of a poly(A) sequence at the 3′ end of an RNA chain could serve to protect this chain from nucleolytic enzymes. As a result, the nuclear RNA segments that have acquired this sequence would be preserved during processing, while the remainder of the nuclear RNA is destroyed. The same mechanism could prevail in the cytoplasm, thus insuring that the mRNA remains intact as long as

it retains a poly(A) segment. There is very little knowledge of the enzymes that may be involved in the degradation of nuclear RNA and of mRNA. The simplest model for the protecting role of poly(A) would be that the major degrading enzyme be a 3′-exonuclease, and that this segment provide a barrier against the action of the enzyme. The actual mechanism of degradation may be more complex, as suggested by the fact that globin mRNA breakdown in frog oocytes occurs only when it is being translated.

The above scheme does not account for the stability of the mRNA species which do not have any poly(A) sequence, such as the histone mRNAs. In this case, one would have to postulate that some other stabilizing structure occurs at the 3′ end of the RNA chains. The change in histone mRNA stability that occurs after cessation of DNA synthesis could then be explained by a modification of such a structure caused by an appropriate cellular signal.

If mRNA degradation in the cytoplasm requires prior loss of the poly(A) sequence, then a process must exist for the controlled removal of this sequence. The proteins bound to the poly(A) might be involved in such a process. They have been shown to protect this sequence from the action nucleases. Moreover, the poly(A)-protein interaction appears to be influenced by other portions of the mRNA. The enzyme or enzymes responsible for the selective degradation of poly(A) that takes place in the cytoplasm are unknown. This process could be promoted either by a highly specific endonuclease, or by a nonspecific exonuclease. The degrading enzyme would have to be able to overcome the protecting effect of the proteins associated with poly(A). There is some indication that a soluble protein factor interferes with the poly(A)-protein interaction, thus rendering this sequence susceptible to enzymic attack.

Another possible mechanism for the removal of poly(A) from the mRNA chains is through endonucleolytic cleavage within the 3′ noncoding region. The potential for diversity in the structure of this region could then provide a basis for differential sensitivity to the enzyme, and consequently for mRNA stability. There is some indication that this region is particularly sensitive to nucleolytic attack. This model, however, would seem to preclude any relation between rate of poly(A) shortening and mRNA stability.

Other roles for poly(A) are possible. Some of the features of mRNA processing in the nucleus tend to suggest that the poly(A) sequence may participate in the splicing event that joins the 3′-terminal segment to the rest of the mRNA. This would require a direct interaction between the poly(A) and the splicing machinery. The possibility of interaction between the poly(A) and the 5′ end of the mRNA has also been raised. This would permit the poly(A) segment to influence the translation process.

Much remains to be done to establish firmly the biological role of the poly(A) sequence, and the results to date indicate that this role may be quite important. The biochemical mechanisms by which this sequence discharges its functions also promise to be quite interesting, and may possibly represent a unique class of biochemical interactions.

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