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Possible Relationship of Poly(A) Shortening to mRNA Turnover

(guanosine chase/emetine)

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ABSTRACT **Whereas the original size of poly(A) in HeLa cells is about 200 nucleotides, at steady state most of the poly(A) in mRNA contains less than 50 nucleotides. An endonucleolytic attack on poly(A) is suggested as the most likely method to accumulate short pieces of poly(A). Both poly(A) shortening and mRNA turnover appear to be inhibited by emetine, a drug that stops translation. It is possible that a random endonucleolytic attack leads to scission of poly(A) to a size below which the mRNA is unstable.**

In mammalian cells, both a portion of hnRNA, the heterogeneously-sized nuclear RNA, some of which is probably precursor to mRNA, and a majority of the cytoplasmic mRNA contain a 3′-terminal poly(adenylic acid) segment (see ref. 1 for review). It was suggested that this poly(A) has some nuclear role in mRNA biogenesis because inhibition of poly(A) synthesis stops the appearance of labeled mRNA in the cytoplasm (1, 2). No role for the poly(A) in the cytoplasm has yet been described. It is apparently not obligatory for protein synthesis since some mRNA molecules lack poly(A) (3–5) and even those mRNA molecules originally containing the poly(A) segment can be translated *in vitro* after its complete or nearly complete removal (6–8).

As tested by gel electrophoresis, the poly(A) attached to hnRNA in HeLa cells appears to have the same size as the poly(A) that initially appears in mRNA (9, 10). However, soon after cytoplasmic appearance, the poly(A) begins to diminish in size, decreasing from about 200 nucleotides to about 50 nucleotides or shorter. At the same time the studies on poly(A) shortening were done, other studies showed that a major fraction of the poly(A) terminated cellular mRNA decayed exponentially with a half-life of many hours (11–13). Since it appeared that the poly(A) required some time for shortening (9, 10) and the mRNA was believed to begin exponential decay upon entering the cytoplasm, it was concluded that mRNA half-life could not be correlated with poly(A) removal (11–13).

In the present experiments we have further analyzed the size and base composition of shorter segments of poly(A) and examined the steady-state distribution of various sizes of poly(A) among mRNA molecules. This distribution is compatible with at least one mode of poly(A) shortening in which the mRNA half-life could be related to the shortening process.

In addition, it has been shown that the apparent turnover of mRNA and the shortening of poly(A) are both retarded by interrupting protein synthesis.

METHODS AND MATERIALS

Cells and Labeling. HeLa cells were grown in suspension in Eagle's medium (14) with [2,8-^{3}H]adenosine (25 mCi/μmol; 0.5–5 mCi for 5×10^7 cells in 25 ml) was used for short-term (5–180 min) labels. For "steady-state" labeling, cells were grown for 4 generations in 15 μM adenine, 0.08 mCi/ml; cell concentration was maintained between 2.5 and 5×10^5 cells per ml. Long labeling (12 hr) with [^{14}C]adenine utilized 0.5 μCi/ml, with a final adenine concentration of 10 μM. For base composition studies, cells at a concentration of 3×10^5/ml were labeled with 0.04 mCi/ml of $^{32}PO_4^{-3}$ for 24 hr in phosphate-free medium containing 5% undialyzed fetal calf serum. [^{3}H]Guanosine pulse-chase was carried out as described (15), with a 10-min exposure of cells to [^{3}H]guanosine (5×10^7 cells in 10 ml, 0.5 mCi per sample) followed by washing in regular medium at 37° and resuspending the cells in medium containing 50 μg/ml of unlabeled guanosine (15).

RNA Extraction. RNA from cytoplasmic extracts prepared either by hypotonic swelling and Dounce homogenization or by NP-40 lysis was obtained by extraction with phenol/chloroform/isoamyl alcohol as described (9, 10).

Poly(A) analysis was carried out by gel electrophoresis of poly(U)-Sepharose-bound RNase-resistant material (16). T1 RNase digestion (10 units/ml) of RNA solutions (in 10 mM Tris·HCl, pH 7.4, 10 mM EDTA at 37°) was followed by addition of NaCl at 0.2–0.4 M and poly(U)-Sepharose selection. After formamide elution, ethanol precipitation, and resolution in 0.2 M NaCl, 10 mM Tris·HCl, 10 mM EDTA, the material was digested with pancreatic RNase (5 μg/ml) for 30 min at 37° before gel electrophoresis on acrylamide gels containing 8 M urea. The gels were sliced and assayed for radioactivity as described (16).

Poly(U)-Sepharose chromatography of undigested RNA molecules was carried out as described (15, 16). Samples were exposed to poly(U)-Sepharose in 0.4 M NaCl containing buffer (0.4 NETS: 0.4 M NaCl, 10 mM Tris·HCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate), followed by a wash with the same buffer lacking NaCl, followed by the same low salt buffer containing 10% formamide to wash free any bound rRNA, and finally by 50% formamide, 50% low salt buffer to elute poly(A)-containing molecules. No rRNA contaminated the bound fraction.

RESULTS

Fig. 1 illustrates the earlier findings that the electrophoretic migration of radioactive nuclear and cytoplasmic poly(A) labeled with [^{3}H]adenosine was the same at the earliest times when cytoplasmic poly(A) can be observed (9, 10); a reduction in size of cytoplasmic poly(A) begins to be detectable within 30 min and is quite pronounced 3 hr.

Both HeLa cell nuclear and cytoplasmic RNA contain short adenylate-rich oligonucleotides, ranging in length from 10 to 50 nucleotides, which are not 3′ terminal, as is the

Abbreviation: hnRNA, heterogeneous nuclear RNA.

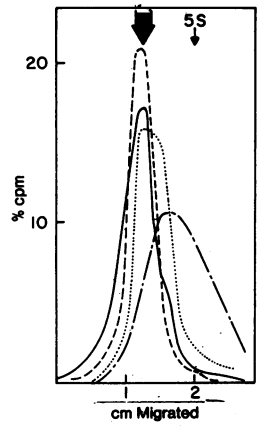

FIG. 1. Electropherograms of poly(A) after various label times. HeLa cells labeled with [^{3}H]adenosine for 10, 30, or 180 min were fractionated. The labeled poly(A) from various samples was subjected to gel electrophoresis along with a ^{32}P-labeled nuclear poly(A) marker (*arrow*) and a 5S [^{14}C]RNA marker. (Only the portions of the gels down to about 50 nucleotides are plotted.) Nuclear poly(A), 10 min (– – –). Cytoplasmic poly(A): 10 min (——); 30 min (·····); 180 min (– — –).

poly(A) segment (17, 18, 20). Most of these "oligoadenylate" regions contain only 60–70% adenylic acid and are resistant to T1 RNase but not to pancreatic RNase (G. Molloy and J. E. Darnell, unpublished observations). Therefore, to see whether the shortened cytoplasmic poly(A) that appeared after prolonged labeling was exclusively composed of adenylic acid, cells were incubated for 24 hr with $^{32}PO_4^{-3}$, and the cytoplasmic poly(A) fraction was prepared from a polyribosomal pellet by phenol extraction and T1 and pancreatic RNase digestion, followed by poly(U)-Sepharose chromatography. The purified poly(A) fraction was separated into size classes

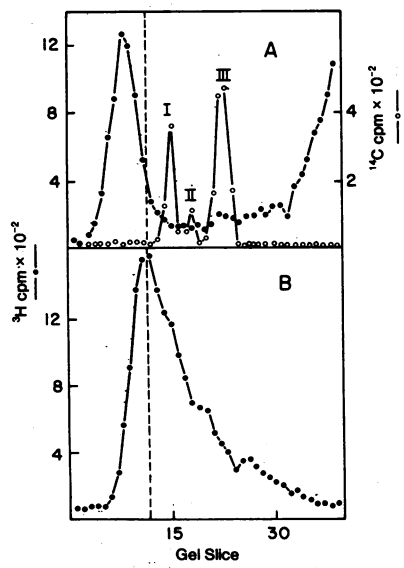

FIG. 2. Electropherograms of "steady-state" nuclear and cytoplasmic poly(A). Poly(A) from the nuclear and cytoplasmic fractions of HeLa cells grown for 3.5 generations in [^{3}H]adenine was subjected to polyacrylamide gel electrophoresis (see *Methods and Materials*). A, nuclear sample; B, post-mitochondrial cytoplasmic sample. *Vertical broken line*, [^{32}P]poly(A) marker used in all gels. I, VA [^{14}C]RNA from adenovirus infected cells; II, 5S HeLa cell [^{14}C]RNA; III, 4S HeLa cell [^{14}C]RNA.

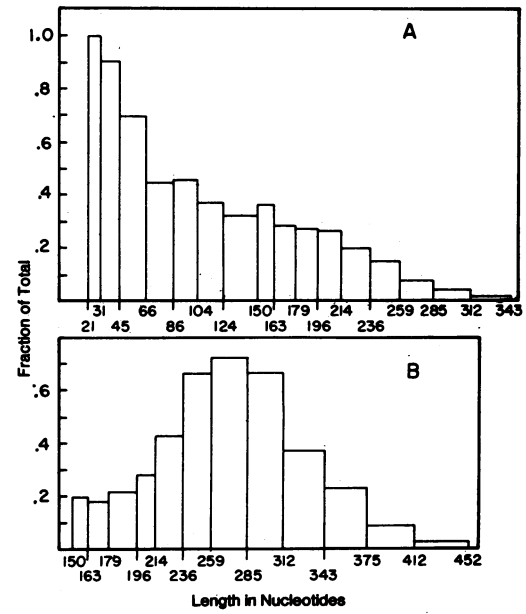

FIG. 3. Size distribution of "steady-state" labeled nuclear and cytoplasmic poly(A). Data from Fig. 2 were used as follows: Poly(A) from each gel slice (or group of slices) was assigned (19, 26) a size range based on migration of markers (VA = 160, 5S = 120, and 4S = 80 nucleotides) (19, 26). The fraction of poly(A) units in each size class was calculated according to the following equation:

$$\left(\frac{\text{cpm}}{\bar{L}} \Big/ \Sigma \frac{\text{cpm}}{\bar{L}}\right) \Big/ \text{Range} = \text{fraction of Total in each size class,}$$

where cpm is radioactivity in each slice (or group of slices), $\bar{L}$ is the median length of poly(A) in each slice, and Range is the number of members of each slice (e.g., an estimated size range of 104–124 has 21 members).

(approximately 120–200, 90–120, 50–90, and 20–50 nucleotides) by gel electrophoresis, and the base composition of each size class was determined. (The gel diagram is not shown, but appeared similar to Fig. 2.) The poly(A) segments above 50 nucleotides were over 99% adenylic acid, with no detectable guanylic or uridylic acid (Table 1). As previously reported, some (about 1%) of the adenylic acid was converted during alkaline hydrolysis to a nucleotide that migrated together with or slightly faster than cytidylic acid, making the apparent presence of cytidylate an unreliable guide of poly(A) purity (17). The 20–50 nucleotide class was at least 95% adenylic acid, with small but definite amounts of uridylic and guanylic

TABLE 1. *Base composition of poly(A) of various sizes*

Estimated size range	% cpm as			
	C	A	G	U
120–200	0.1	99.9	0	0
90–120	0.8	99.2	0	0
40–90	0.8	99.2	0	0
20–48	1.8	95.2	0.8	1.6

^{32}P-Labeled poly(A) purified from cells labeled for 24 hr was subjected to gel electrophoresis, and size of various classes was estimated with markers (see Fig. 2). Base composition on alkaline hydrolysates of the poly(A) eluted from various regions of the gel was determined.

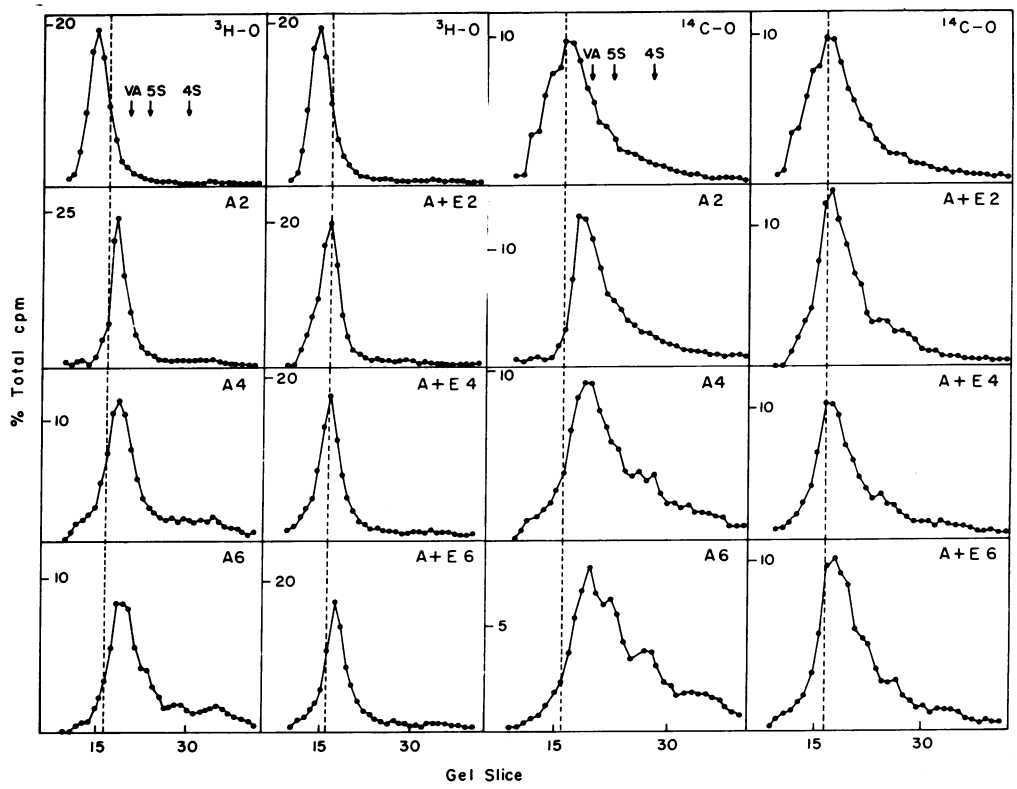

FIG. 4. Effect of emetine on poly(A) shortening during actinomycin D "chase." Cells were labeled with [^{14}C]adenine for 12 hr and [^{3}H]adenosine for 30 min. A sample was taken and actinomycin D (5 μg/ml) was added; the culture was immediately divided and emetine (50 μg/ml) added to one half. Samples were then taken at 2, 4, and 6 hr. Cytoplasmic poly(A) was analyzed by acrylamide gel electrophoresis, and the data are presented as follows: ^{3}H, columns 1 and 2; ^{14}C, columns 3 and 4. Columns 1 and 3 were samples without emetine; columns 2 and 4 samples with emetine. The vertical dashed line represents a ^{32}P marker present in all the gels; VA, 5S, and 4S RNA, all ^{14}C-labeled, were also run with the ^{32}P marker.

acid as well as material migrating as cytidylic acid. Thus, only the poly(A) segments larger than 50 were definitely homopolymers, i.e., exclusively the 3'-terminal poly(A). It seems unlikely that the shortening process would abruptly stop at 50 nucleotides; segments below this size probably represent a mixture of 3'-terminal poly(A) and contaminating oligonucleotides containing the other bases. Further purification would be necessary to prove this point.

The size distribution of poly(A) segments in cells labeled to steady state was determined as the next step in examining the shortening process. Poly(A) from the nucleus and cytoplasm was isolated from cells grown for 3–4 generations in [^{3}H]-adenine. The gel electrophoretic pattern (Fig. 2) showed the cytoplasmic poly(A) to be distributed in a broad size range smaller than 50 to longer than 200 nucleotides, as was seen in the ^{32}P-labeled poly(A) after a 24 hr label. The nuclear poly(A), however, had a major peak of radioactivity in the region of about 250 nucleotides.

The distribution of radioactivity in gel electropherograms of poly(A) does not coincide directly with the numerical distribution of poly(A) units. Migration of RNA molecules through acrylamide gels is a function of the logarithm of the molecular weight (19). Thus, a slice from near the top of a gel (negative pole) will contain a larger spread of sizes of poly(A) than will a slice from near the bottom (positive pole). Furthermore, molecules contribute radioactivity in direct proportion to their length. By assigning size ranges to the poly(A) in each gel slice based on the migration of three markers (20–22), the radioactivity in each slice of the gel electropherograms (Fig. 2) was converted into histograms (Fig. 3) representing the relative frequency of occurrence of each individual size of poly(A). The nuclear poly(A) (Fig. 3B) showed a symmetrical peak with a mean of about 260 nucleotides. Although the nuclear sample contained some material migrating in the region of 20–100 nucleotides (slices 15–35, Fig. 2A), this material (labeled with ^{32}P) did not exceed 60–70% adenylic acid in composition, i.e., it is not poly(A). In the cytoplasmic sam-

TABLE 2. *% Poly(A) of various sizes*

	^{3}H, actinomycin				^{14}C, actinomycin			
Size range	0	2	4	6	0	2	4	6
			No emetine					
163–200	65.1	17.6	14.6	5.3	41.2	37.1	34	24.5
109–162	9.4	41.6	32.8	32.5	27.5	30.9	29.1	36
49–108	15.3	31.5	39.1	44.6	24.2	26.5	28.9	31.2
32– 48	10.2	9.3	13.4	17.6	7.1	5.5	7.9	8.3
			Plus emetine					
163–200		38.3	26.6	16.6		62.6	53.2	40.3
109–162		29.7	28.3	36.8		13.8	16.4	24.7
49–108		23.5	31.7	30.7		17.2	21	23.9
32– 48		8.5	13.4	15.9		6.4	9.4	11.1

Data from Fig. 4 were used to calculate (similarly to Fig. 3) % of poly(A) units found in various size ranges after actinomycin D chase or actinomycin D plus emetine. ^{3}H, actinomycin indicates 30-min [^{3}H]adenosine label, actinomycin chase for 2, 4, or 6 hr; ^{14}C indicates 12-hr [^{14}C]adenine label.

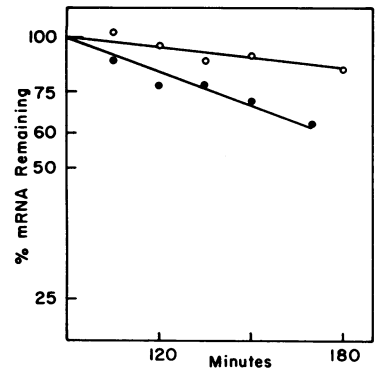

FIG. 5. Effect of emetine on decay of mRNA during actinomycin chase. mRNA from cells pulse-labeled and chased with guanosine (10-min label; chase to 50 min before first sample) was measured by chromatography on poly(U)-Sepharose (15). Actinomycin was added at 60 min to half the culture (●) and actinomycin plus emetine (○) to the other half. The mRNA in the sample withdrawn at 60 min was taken to be 100%.

ple, however, the percentage of poly(A) units in each size class rose with decreasing size. Thus, a high proportion of mRNA molecules at steady state is terminated with considerably shortened poly(A). The lower limit in the distribution of poly(A) size is undetermined since the shortest segments of adenine-labeled RNase-resistant material migrated in the region of the gel where the base composition studies indicated the presence of some nucleotides other than adenylic acid. The significance of these analyses will be detailed in the *Discussion* section.

The shortening of cytoplasmic poly(A) was not confined to any particular class of cytoplasmic structures. Similar poly(A) profiles were found from 60 to 90S structures and the total cytoplasmic extracts (9, 10) regardless of the duration of the label or chase period.

As previously described (9, 10), the most effective means of studying the time-course of shortening of poly(A) was to treat labeled cells with actinomycin D; previously labeled poly(A) was observed then to decrease in size (9, 10). This reduction in poly(A) size was previously reported to be unaffected in cells treated with emetine, a drug that inhibits protein synthesis by over 95% (23). This earlier conclusion was based on the migration of the major peak of radioactive poly(A) and not the total distribution of poly(A) units. With the realization that the steady-state distribution shown in Fig. 3 really represented a preponderance of very short poly(A) chains, a more thorough examination of the poly(A) shortening in emetine was undertaken. Cells were doubly labeled by 12 hr of growth in [^{14}C]adenine and 30 min of growth in [^{3}H]adenosine. The culture was divided after the addition of actinomycin D to prevent further RNA synthesis, and one-half was treated with emetine. Samples were taken from both cultures; the poly(A) was purified and compared by gel electrophoresis to the poly(A) in samples at the time of actinomycin D addition. Fig. 4 and Table 2 show that about 25% of the ^{3}H was in poly(A) units shorter than 100 nucleotides and over 65% was in units longer than 163 nucleotides. During the ensuing 6-hr incubation, the [^{3}H]poly(A) in units longer than 163 nucleotides decreased to about 5% and that in the smaller units increased to 63%. This shortening process was substantially diminished in the culture treated with emetine. The peak of total [^{3}H]poly(A) shifted more slowly to a smaller size, and much less of the very short poly(A) appeared in the emetine-treated culture by 6 hr.

The same general result was obtained from an analysis of the long-labeled [^{14}C]poly(A); the control contained poly(A) of all sizes, the average distribution resembling the steady state. The long-labeled poly(A) in the actinomycin-treated culture showed a rapid loss of the largest poly(A) and an accumulation of a greater amount of shorter poly(A) segments. Both these changes were retarded by emetine. Thus, our earlier conclusion (9, 10) about the failure of emetine to stop poly(A) shortening was wrong. Both new and old poly(A) segments were shortened in actinomycin-treated cells, and this shortening process was slowed but not entirely prevented by emetine.

Studies with bacteria some years ago demonstrated that if ribosomal movement, and therefore protein synthesis, was inhibited by treatment with chloramphenicol or anaerobiosis, then mRNA decay was retarded (24). Emetine also stops protein synthesis by blocking ribosomal movement in mammalian cells (23) and, therefore, might also slow down mRNA turnover. Furthermore, since emetine retarded poly(A) shortening, if it also retarded mRNA turnover, a link between the two processes might be inferred.

Recently a re-examination of mRNA (15) turnover in cells briefly labeled with guanosine has allowed the observation of mRNA of shorter half-life than earlier experiments (11–13). All of these studies have been made possible by the ability to accurately assay poly(A)-terminated mRNA by column chromatography (25). The disappearance of guanosine label from mRNA during a chase with unlabeled guanosine and actinomycin was determined in the presence and absence of protein synthesis. When cells were making protein normally, they lost 38% of their labeled mRNA within 2 hr after chase. In the presence of emetine, the decay of mRNA is only half as fast. The older experiments with bacteria also show about a 50% protection of mRNA from decay by slowing protein synthesis (24).

A caution must be raised about mRNA turnover studies in which disappearance of radioactive mRNA from a chromatographic fraction selected by poly(A) content is equated with mRNA turnover (11–13, 15). If the poly(A) were shortened below the length required to bind the whole molecule, the mRNA would be undetected; there would be no assurance that the whole molecule had been turned over. We have determined (Puckett, Georgieff, and Darnell, unpublished) that mRNA molecules containing poly(A) of more than 50 nucleotides binds to poly(U)-Sepharose quantitatively (>95%); those containing poly(A) of from 30 to 50 nucleotides bind approximately 60%, and those with poly(A) less than 30 nucleotides down to the shortest measurable poly(A) bind about 50%. Since, in the experiments of Fig. 4 and Table 2, 87% of the poly(A) from mRNA molecules remaining after 4 hr of actinomycin chase was still larger than 50 nucleotides, we don't believe the loss (presumed turnover) of 38% of the guanosine-labeled mRNA molecules in Fig. 5 would be due to poly(A) shortening alone. It appears likely then that both poly(A) shortening and mRNA turnover are inhibited by emetine.

DISCUSSION

In considering models for poly(A) shortening and mRNA turnover and the possible relationship between the two, there

are two experimental results that must be accommodated: (*i*) mRNA initially contains a poly(A) segment of about 200 nucleotides, while the steady-state distribution of poly(A) favors much shorter poly(A) units (Figs. 2 and 3), and (*ii*) poly(A)-containing mRNA appears to decay exponentially (11–13). Further, there is an important logical consequence of exponential mRNA disappearance: since molecules of all ages are lost by turnover to be replaced only by new molecules, there would necessarily be more molecules between 0 and 30 min old than molecules between 30 and 60 min old, etc.

There are two possible enzymatic modes of poly(A) destruction—*exonucleolytic* and *endonucleolytic*. Likewise, mRNA turnover and the process of poly(A) shortening either *are* or *are not* related. The purpose of this discussion is to present the logic for or against a specific type of enzymatic cleavage of poly(A) and the possible relationship of mRNA turnover to the activity that shortens poly(A), keeping in mind the constraints given in the paragraph above.

Exonucleolytic destruction of poly(A), where each adenylate residue was equally likely to be removed, would not result in the accumulation of molecules bearing short poly(A). Only if exonucleolytic cleavage slowed down for the shorter poly(A) segments would it be possible to accumulate mRNA molecules bearing short poly(A) as the predominant species. In either case of exonucleolytic poly(A) destruction, however, there would be a time lag between the entry of an mRNA molecule into the cytoplasm and the extreme shortening or disappearance of its poly(A). Exponential decay of mRNA, therefore, could not be related to such a shortening mechanism. A variable, decreasing exonucleolytic poly(A) destruction which is not causally related to mRNA turnover is, however, compatible with present results.

Random *endonucleolytic* cleavage of poly(A) would result in the accumulation of short poly(A) segments. In addition, if some minimal length of poly(A) were critical to maintaining mRNA stability, destruction to or below that length by an endonuclease could be a random event. Therefore, random endonucleolytic poly(A) cleavage could be responsible for both exponential mRNA decay and the accumulation of short poly(A) segments. It is, of course, possible that endonucleolytic poly(A) shortening and mRNA decay might proceed in parallel but be unrelated.

However, it is clear from this analysis of the size distribution of poly(A) that random destruction of mRNA could be determined by a random endonucleolytic destruction of poly(A) because short poly(A) is the predominant size class at steady state. Furthermore, when mRNA half-life is lengthened by stopping protein synthesis, poly(A) shortening is greatly slowed. It is possible that poly(A) functions in the cell cytoplasm to help determine mRNA half-life.

Eventual solution of the possible interrelationship of poly(A) shortening and mRNA turnover may require that either a number of specific mRNAs with distinct but different half-lives or a single specific mRNA whose half-life could be physiologically varied be analyzed for poly(A) size as a function of age.

A first step in this direction appears to have been taken with the recently reported results of Huez *et al.* (8). They found that the stimulation of hemoglobin production in *Xenopus* ooctyes by hemoglobin mRNA from which poly(A) was enzymatically removed was of shorter duration than the stimulation by native hemoglobin mRNA.

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