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# Polyadenylic Acid Sequences on 3′ Termini of Vaccinia Messenger Ribonucleic Acid and Mammalian Nuclear and Messenger Ribonucleic Acid†

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ABSTRACT: The location of poly(A) on vaccinia mRNA and HeLa n- and mRNA was determined with the use of polynucleotide phosphorylase. This enzyme, which phosphorylizes RNA in a 3′→5′ direction, degraded the poly(A) moiety before the rest of the RNA. When the poly(A) was first removed from the RNA by ribonuclease digestion, then submitted to phosphorolysis, virtually all the poly(A) was degraded. This indicated that all poly(A) sequences contained 3′-hydroxyls, which are characteristic of RNA 3′ termini. The same results were obtained when the poly(A) was degraded by a mammalian 3′-exoribonuclease which hydrolyzes only RNA chains bearing 3′-hydroxyls. The localization of poly(A) on the 3′ terminus of vaccinia mRNA was also supported by the kinetics of addition of the poly(A) to viral RNA being synthesized *in vitro*.

The existence of poly(A) sequences in eukaryotic cells has been recognized for several years (Hadjivassiliou and Brawerman, 1966; Edmonds and Caramela, 1969). The covalent attachment of poly(A) to messenger RNA (mRNA) was demonstrated recently, first for vaccinia mRNA (Kates and Beeson, 1970) and subsequently for other viral (Philipson *et al.*, 1971; Lai and Duesberg, 1972; Weinberg, *et al.*, 1972)[1] and animal cell nuclear and mRNAs (Kates, 1970; Lim and Canellakis, 1970; Darnell *et al.*, 1971b; Edmonds *et al.*, 1971; Lee *et al.*, 1971). Presently very little is known about how the poly(A) is synthesized, or what function it serves. To shed light on these problems it is important to know where on the RNA the poly(A) sequence is located.

In 1970, Kates reported an experiment which indicated that poly(A) is on the 3′ end of vaccinia mRNA. Poly(A), when released from the RNA by $T_1$ and pancreatic ribonuclease digestion, contained 1 adenosine and 0.2 adenosine tetraphosphate per 110 adenosine 3′-monophosphates. Because the presence of the adenosine could be accounted for only if the poly(A) was 3′ terminal, it appeared that most poly(A) sequences were on the 3′ ends of RNA. The low amounts of adenosine tetraphosphate might have been due to some poly(A) on the 5′ end of RNA, or due to the existence in that system of some free poly(A). Further experiments were needed to clear up this ambiguity.

While the work presented below was in progress, the following information regarding poly(A) location was reported.

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Burr and Lingrel (1971) determined that there are five or six adenosine residues on the 3′ end of globin mRNA. Mendecki *et al.* (1972) detected labeled adenosine in alkaline hydrolysates of poly(A) from sarcoma 180 cells and concluded that the poly(A) is at the 3′-hydroxyl terminus of at least some of the RNA molecules. Recently, Molloy *et al.* (1972) used a 3′-exoribonuclease to demonstrate that at least 73% of the poly(A) from HeLa polysomal RNA, and 90% of the poly(A) from L cell polysomal RNA, is on the 3′ end of the RNA. On the other hand, Ryskov *et al.* (1972) have concluded that poly(A) is at the 5′ end of nuclear RNA (nRNA) molecules from ascites cells.

We have conducted a variety of experiments to demonstrate that virtually all of poly(A) on vaccinia mRNA is on the 3′ end. Moreover, we have found that at least 97.6% of the poly(A) from HeLa mRNA and heterogeneous nRNA and at least 88% of the poly(A) from L cell nRNA is on the 3′ end of those molecules.

## Materials

[$^3$H]Poly(A) was purchased from Miles Laboratories. Poly(U) was purchased from either Miles Laboratories or Sigma Chemical Co. Electrophoretically purified deoxyribonuclease, pancreatic ribonuclease, and ribonuclease $T_1$ were purchased from Worthington Biochemical Corp. Polynucleotide phosphorylase type 15 was obtained from P-L Biochemicals. Ehrlich ascites tumor cell nuclear exoribonuclease, a generous gift of Dr. Michael Sporn, was purified as described in Molloy *et al.* (1972).

## Methods

*Preparation of Virus and Viral Cores.* The WR strain of vaccinia virus was grown in HeLa $S_3$ cells in spinner culture (McAuslan, 1963). The virus was prepared by a modification (Kates and Beeson, 1970) of the method of Joklik (1962). Viral cores were prepared by the modified method (Sheldon *et al.*, 1972) of Kates and Beeson (1970).

*Preparation and Use of Poly(U) Fiber Glass Filters.* Poly(U) filters were prepared and used as described by Sheldon *et al.* (1972). Variation of the pH of the binding buffer up to pH 9 and inclusion of 0.5% sodium dodecyl sulfate in the buffer had no effect on the retention of poly(A) or RNA by the filters.

*Ribonuclease Assay for Poly(A).* Ribonuclease resistance of RNA was determined by digesting the RNA with 10 μg/ml of pancreatic ribonuclease and 1 μg/ml of ribonuclease $T_1$ in 0.01 M Tris-HCl (pH 7.5)–0.3 M KCl for 20 min at 37°.

*Preparation of HeLa RNA.* HeLa $S_3$ cells ($2 \times 10^8$) were incubated for 30 min at 37° in spinner culture in F-14 medium supplemented with 5% calf serum–0.04 μg/ml of actinomycin D–1 μg/ml of ethidium bromide–$10^{-5}$ M cytosine arabinoside. After being labeled with 4 mCi of [$^3$H]adenosine (New England Nuclear, 15.5 Ci/mmole) for 2 hr the cells were poured into ice-cold phosphate-buffered saline, washed once in this solution, and resuspended at $10^8$ cells/ml in 10 mM Tris-HCl (pH 8.5)–150 mM NaCl–1.5 mM $MgCl_2$ at 4°. The cells were lysed by the addition of nonidet NP-40 (Shell Oil Co.) to a concentration of 0.5%. The cytoplasmic fraction was layered on 10 ml of 7–47% (w/w) sucrose gradients buffered with 10 mM Tris-HCl (pH 8.5)–50 mM $MgCl_2$–0.5 M NaCl and centrifuged in the Beckman SW 41 rotor for 110 min at 39,000 rpm at 5°. The polysomal RNA was extracted twice with phenol–chloroform (1:1, v/v) (Perry *et al.*, 1972). The aqueous phases were pooled, precipitated from 2 volumes of ethanol, and reprecipitated from 2 M LiCl. This frees the RNA of DNA, RNA smaller than ~5–6 S, and mononucleotides (Penman *et al.*, 1968).

The HeLa cell nRNA was prepared essentially according to Wall and Darnell (1971). The nuclei were resuspended at $5 \times 10^7$ per ml in 50 mM sodium acetate (pH 5.1)–10 mM EDTA–0.5% sodium dodecyl sulfate, broken in a Dounce homogenizer, and extracted at 45° with phenol–chloroform–isoamyl alcohol. The aqueous phase was reextracted twice with phenol–chloroform–isoamyl alcohol, precipitated twice from 2 M LiCl and once from ethanol and was stored as an ethanol precipitate at −20°.

*Preparation of Vaccinia RNA.* Conditions for RNA and poly(A) synthesis *in vitro* by cores were described by Kates and Beeson (1970). However, the RNA was extracted with chloroform–phenol (1:1, v/v) according to the method of Perry *et al.* (1972). "Early" and "late" vaccinia RNA were prepared from whole cytoplasm. Early RNA was labeled with [$^3$H]adenosine from 65 to 85 min postinfection in the presence of $10^{-5}$ M cytosine arabinoside, while late RNA was labeled from 195- to 210-min postinfection (Kates and McAuslan, 1967). The RNA was extracted three times with chloroform–phenol and precipitated with 3 volumes of ethanol.

*Preparation of L Cell Nuclear Poly(A).* RNA was extracted as described by Greenberg and Perry (1971) from a nucleoplasmic fraction of L cells which were incubated for 100 min with 0.08 μg/ml of actinomycin and labeled during the latter 60 min with 5 μCi/ml of [$^3$H]adenosine. Poly(A) fragments were excised from this RNA by a 1-hr incubation at 37° with 2 μg/ml of pancreatic ribonuclease in the presence of 0.27 M NaCl, and the poly(A) was purified by adsorption and elution from Millipore filters (see Molloy *et al.*, 1972, for further details).

*Incubations with Ascites Nuclear Exoribonuclease.* Conditions for hydrolysis of poly(A) preparations by ascites cell exoribonuclease and assay of the extent of hydrolysis of L cell nuclear poly(A) were as described previously (Molloy *et al.*, 1972).

## Results

*Kinetics of Attachment of Poly(A) to RNA by Vaccinia Cores in Vitro.* Vaccinia cores synthesize *in vitro* mRNA which contains poly(A) sequences (Kates and Beeson, 1970). RNA synthesis proceeds in a 5′→3′ direction. Thus, if the poly(A) is on the 5′ end or internally located in a completed RNA chain, then at any given time some of the growing RNA chains should contain poly(A) sequences. Conversely, if the poly(A) is on the 3′ end of completed RNA, then growing RNA chains will acquire poly(A) only after the RNA chains have been completely synthesized.

Vaccinia cores were allowed to synthesize RNA for 10 min, in order to randomize chain initiation, elongation, and termination. [$^3$H]UTP was then added, and at short intervals thereafter samples were removed, and the reaction was stopped with 0.01 M EDTA–0.5% sodium dodecyl sulfate. Each sample was assayed for total trichloroacetic acid precipitable [$^3$H]-RNA, and for [$^3$H]RNA that could be bound to a poly(U) fiber glass filter. These filters retain only poly(A) and RNA that contains poly(A). As discussed above, if poly(A) is on the 3′ end of mRNA, then only completed chains will contain poly(A), and thus only completed chains will be retained on poly(U) filters. Because under these conditions chain growth is asynchronous, at any particular time the synthesis of very few chains will be nearing or at completion. Thus if

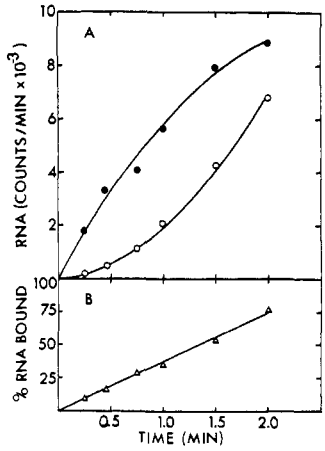


FIGURE 1: Kinetics of attachment of poly(A) to RNA by viral cores. Cores ($1.4 \times 10^{11}$) were incubated for 5 min at 37° in 2.8 ml of 0.1 M Tris-HCl (pH 8.5)–10 mM mercaptoethanol–5 mM $MgCl_2$–1.25 mM ATP–1.25 mM GTP–1.25 mM CTP–0.05 mM UTP, at which point 14 μCi of [$^3$H]UTP (20 Ci/mmole, New England Nuclear) was added. At intervals thereafter, 0.4-ml aliquots were removed and pipetted into 1.6 ml of 1.25 mM EDTA–0.63% sodium dodecyl sulfate–150 mM NaCl to inhibit the reaction and lyse the cores. The aliquots were assayed by precipitating 0.9 ml on nitrocellulose filters with cold 5% trichloroacetic acid, and by passing 0.9 ml through a poly(U) filter. The filters were rinsed and counted as described by Sheldon *et al.* (1972). The results in part A are expressed as (●), acid-precipitable label; (○), label retained on poly(U) filters. (B) (△) Per cent of acid-precipitable RNA that bound to poly(U) filters.

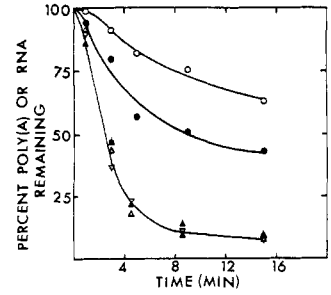


FIGURE 2: Degradation of core-synthesized RNA and its poly(A) moiety by polynucleotide phosphorylase. RNA synthesized *in vitro* by cores and labeled with [$^3$H]AMP (100 Ci/mole) and [$^{14}$C]-UMP (6 Ci/mole) was purified, precipitated twice from 2 M LiCl, and dissolved in 0.1 ml of 10 mM Tris-HCl (pH 7.5). Phosphorolysis was carried out at 37° in a volume of 0.63 ml containing 0.5 unit/ml of polynucleotide phosphorylase–0.1 M Tris-HCl (pH 8.5)–5 mM $MgCl_2$–10 mM sodium phosphate. At the indicated times, 0.1-ml samples were removed from the reaction and pipetted into 0.5 ml of 12.5 mM EDTA–0.15 M NaCl. One-third of each sample was precipitated with 5% trichloroacetic acid onto nitrocellulose filters, one-third assayed for ability to bind to poly(U) filters, and one-third was assayed for ribonuclease-resistant material. The initial material present and symbols used are: (●), acid-insoluble [$^3$H]-AMP, 10,770 cpm; (—○—), acid-insoluble [$^{14}$C]UMP, 1279 cpm; (○), [$^3$H]AMP retained on poly(U) filters, 12,151 cpm; (△), [$^{14}$C]-UMP retained on poly(U) filters, 1307 cpm; (▽), ribonuclease-resistant [$^3$H]AMP, 3800 cpm.

poly(A) is on the 3′ end of completed chains, very little of the [$^3$H]UTP immediately incorporated into RNA should be in RNA that contains poly(A). This proportion should increase with time as RNA chains are completed.

Figure 1A shows that immediately after the [$^3$H]UTP was added, much more label entered total acid-precipitable RNA than RNA that could be bound to poly(U) filters. Figure 1B expresses the amount of RNA retainable by the poly(U) filters as a percentage of the total RNA. It is apparent that initially very little, if any, of the label entered RNA which contained poly(A) sequences. With time the proportion of labeled RNA that contained poly(A) increased greatly. The simplest explanation is that after RNA chains are completed they acquire poly(A) sequences on their 3′ ends.

*Exonucleolytic Degradation of Vaccinia RNA.* The hypothesis that poly(A) is on the 3′ end of mRNA gives rise to two predictions. The first is that the 3′ end of the RNA should be richer in AMP and poorer in UMP, CMP, and GMP than is the molecule as a whole. Thus, during the initial stages of 3′-exonucleolytic degradation of the RNA a higher proportion of the total AMP than of the total UMP should be released from the RNA. The second prediction is that incubation of the RNA with a 3′-exonuclease should degrade the poly(A) before the rest of the RNA. The poly(A) may be assayed in two ways: by its resistance to pancreatic and $T_1$ ribonucleases, and by the ability of poly(A) and RNAs that contain poly(A) to bind to poly(U) fiber glass filters. Therefore, 3′-exonucleolytic degradation of the RNA should very rapidly degrade the RNase-resistant poly(A) and render the remaining undegraded RNA unable to bind to poly(U) filters.

Polynucleotide phosphorylase was used as the 3′-exonuclease. In the presence of inorganic orthophosphate, this enzyme phosphorylizes RNA in a 3′→5′ direction, releasing nucleoside 5′-diphosphates (Grunberg-Manago and Ochoa, 1955). For these experiments to be valid it is essential that the phosphorylase used contained no exo- or endonuclease contaminants. Because the phosphorylase requires phosphate for phosphorolysis, one can assay for contaminating nucleases by determining if RNA is degraded when incubated with the phosphorylase in the absence of phosphate. No acid-precipitable material was released from either Miles [$^3$H]poly(A) or vaccinia [$^3$H]RNA when they were incubated with polynucleotide phosphorylase in the absence of phosphate for 20 min. Therefore, the polynucleotide phosphorylase used did not contain any gross nuclease contaminants. The possibility of trace endonuclease contaminants remained, but endonucleases would rapidly cleave the poly(A) moiety from the rest of the RNA, making the RNA incompetent to bind to poly(U) filters. However, [$^{14}$C]UMP-labeled vaccinia RNA, when incubated with the phosphorylase in the absence of phosphate, lost none of its ability to bind to poly(U) filters. On the basis of these controls it appeared that the phosphorylase used was free of exo- or endonuclease contaminants.

If poly(A) is on the 3′ end of RNA, then phosphorolysis of the RNA should result in a more rapid loss of acid-precipitable AMP than UMP from the RNA. To test this, RNA was synthesized by cores and labeled with [$^{14}$C]UMP and [$^3$H]AMP. The RNA was precipitated from 2 M LiCl to free it of nucleotides and any free poly(A) which might be present (Penman *et al.*, 1968; R. Sheldon, unpublished results). All of this RNA could be bound to poly(U) filters, indicating that all RNA chains contained poly(A) sequences. The RNA was then incubated with the phosphorylase and phosphate. Samples were removed at various times and assayed for $^{14}$C- and $^3$H-labeled, acid precipitable material. Figure 2 shows that phosphorolysis resulted in the immediate loss of some [$^3$H]-AMP, while there was a slight lag before much [$^{14}$C]UMP was lost. At all times during the reaction more AMP than UMP had been lost. This indicates that the 3′ end of the

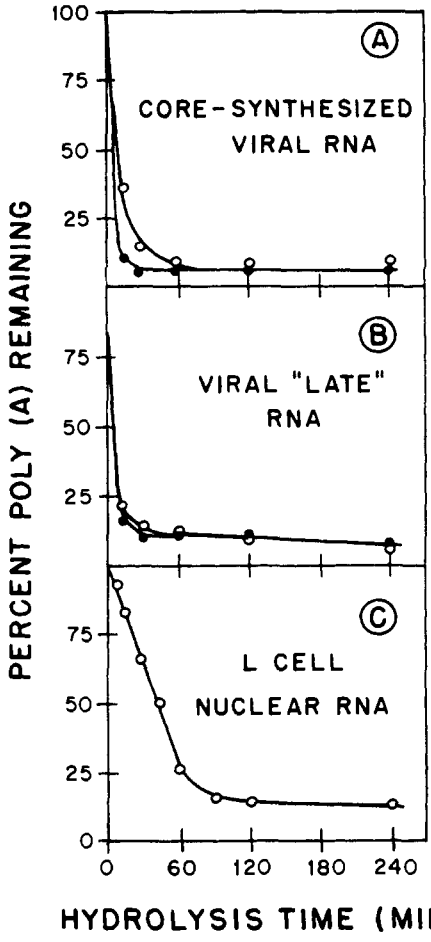


FIGURE 3: Hydrolysis of poly(A) with ascites nuclear exonuclease. $^3$H-Labeled poly(A) fragments were excised with ribonuclease from (A) RNA synthesized *in vitro* by viral cores; (B) viral "late" RNA; and (C) L cell nuclear heterogeneous RNA, and incubated at 37° with 1 unit/ml of nuclear exonuclease. At various times aliquots were withdrawn, and the poly(A) remaining was measured by adsorption to either (A and B) poly(U) fiber glass filters or (C) Millipore filters (Lee *et al.*, 1971). In panels A and B the solid circles (●) indicate parallel experiments in which an equivalent portion of poly(A) was incubated for 30 min at 24° with 50 μg/ml of *Escherichia coli* alkaline phosphatase before incubation with the exoribonuclease (see Molloy *et al.*).

RNA is considerably richer in AMP than UMP, and is consistent with a poly(A) sequence being on the 3′ end.

Poly(A) may be detected more directly by its resistance to RNase degradation, and by the ability of RNAs that contain poly(A) to bind to poly(U) filters. If poly(A) is on the 3′ end of mRNA, then phosphorolysis of the mRNAs should result in a more rapid loss of the poly(A) than the rest of the RNA. Therefore, the amount of RNase-resistant, AMP-labeled RNA and the amount of RNA that can bind to poly(U) filters should decrease more rapidly than the total amount of acid-precipitable RNA. [$^{14}$C]UMP-, [$^3$H]AMP-labeled mRNA was incubated with the phosphorylase, and at various times during phosphorolysis samples were removed and assayed for acid-precipitable RNA, ribonuclease-resistant poly(A), and for the ability of the RNA to bind to poly(U) filters. Figure 2 shows that the ability of both [$^{14}$C]UMP- and [$^3$H]-AMP-labeled RNA to bind to poly(U) filters decreased at the same rate as the amount of RNase-resistant poly(A). The amount of poly(A) decreased much more rapidly than the amount of acid-precipitable RNA. After 5 min, about 80% of the poly(A) had been phosphorylized while only about 20% of the [$^{14}$C]UMP-labeled material and 40% of the [$^3$H]AMP-labeled material had been degraded. The more rapid loss of poly(A) than RNA during phosphorolysis suggests that much of the poly(A) is at or near the 3′ end of the RNA.

*Degradation of Poly(A) by Ascites Nuclear 3′-Exoribonuclease.* The data presented above indicate that most of the poly(A) is near the 3′ terminus. It does not demonstrate whether the poly(A) is directly at the 3′ end, nor does it eliminate the possibility that some poly(A) is elsewhere on the RNA, perhaps at the 5′ end. To resolve this, poly(A) was first removed from the RNA by RNase digestion. Only poly(A) sequences that had been directly on 3′ termini will retain 3′-hydroxyls, while those from elsewhere on the RNA will have 3′-phosphates. The purified poly(A) sequences were then incubated with Ascites tumor nuclear 3′-exonuclease, which degrades RNA chains bearing 3′-hydroxyls but not 3′-phosphates (Sporn *et al.*, 1968). Thus, only poly(A) sequences that had been directly on the 3′ end of RNA will be degraded by the enzyme.

The data in Figure 3A,B show that poly(A) prepared from RNA synthesized *in vitro* by cores and poly(A) prepared from RNA synthesized *in vivo* "late" after infection (after viral DNA synthesis commences) were at least 95% sensitive to hydrolysis by the enzyme. Preincubation with alkaline phosphatase to remove any 3′-phosphates did not significantly increase the proportion of the poly(A) susceptible to the enzyme. These results demonstrate that at least 90–95% of vaccinia poly(A) is directly on the 3′ terminus of viral mRNA.

Figure 3C depicts a similar experiment with poly(A) fragments excised with pancreatic ribonuclease from the heterogeneous nRNA of mouse L cells. At least 88% of this poly(A) was susceptible to hydrolysis by the exoribonuclease, a result comparable to that obtained with poly(A) derived from L cell mRNA (Molloy *et al.*, 1972).

*Degradation of Poly(A) by Polynucleotide Phosphorylase.* Bacterial polynucleotide phosphorylase is similar to the nuclear 3′-exoribonuclease in that both will degrade RNAs bearing 3′-hydroxyls, while neither will degrade RNAs bearing 3′-phosphates. Therefore, the experiment described in the preceding section may be performed with polynucleotide phosphorylase. Only poly(A) that had been on the 3′ terminus of mRNA will be susceptible to polynucleotide phosphorylase degradation; poly(A) from other regions of the RNA will bear 3′-phosphates, and hence be resistant to the phosphorylase. Any contaminating phosphatase activity would give erroneous results, since it would cleave 3′-phosphates to 3′-hydroxyls. Several experiments were performed to eliminate the possibility that any phosphatase was present.

Alkaline phosphatase can cleave *p*-nitrophenyl phosphate to nitrophenol and phosphate (Garen and Levinthal, 1960). Incubation of *p*-nitrophenyl phosphate with the phosphorylase for 5.5 hr resulted in the release of absolutely no nitrophenol.

In the absence of salt, pancreatic ribonuclease can degrade poly(A) to fragments bearing 3′-phosphates (Beers, 1960). The results in Table I demonstrate that after a 15-min incubation with 10 μg/ml of pancreatic ribonuclease, only 26% of the poly(A) fragments were sensitive to the phosphorylase. However, the fragments when preincubated with alkaline phosphatase were over 93% sensitive to polynucleotide phosphorylase.

If any 3′-phosphates are present, then pretreatment of the sample with alkaline phosphatase should increase the rate and/or extent of subsequent phosphorolysis with the phosphorylase. In all experiments reported in Table I, one-half

TABLE I: Sensitivity of Poly(A) to Degradation by Polynucleotide Phosphorylase.[a]

| Source of Poly(A) | Poly(A) Phosphorylized (%) | |
|---|---|---|
| | −Alkaline Phosphatase Preincubation | +Alkaline Phosphatase Preincubation |
| Viral RNA synthesized by cores | 99.3 | 98.3 |
| Viral "early" RNA | 94.6 | 96.6 |
| Viral "late" RNA | 96.5 | 90.2 |
| HeLa polysomal RNA | 97.6 | 96.7 |
| HeLa nuclear RNA | 99.7 | 92.4 |
| Poly(A) degraded by pancreatic ribonuclease[b] | 25.9 | 93.1 |

[a] The preparation of the poly(A) and subsequent phosphorolysis were as described for Figure 4. Samples were phosphorylized for 90 min, and all but the nuclear poly(A) assayed by acid precipitation. Because some nonpoly(A), ribonuclease-resistant RNA was present in the nuclear poly(A) preparation, this poly(A) was assayed by filtration through poly(U) filters. [b] Poly(A) was partially degraded with 10 μg/ml of pancreatic RNase in 0.01 M Tris-HCl (pH 7.5) for 15 min at 37° to produce fragments containing 3′-terminal phosphates. The fragments were phenol-extracted, precipitated with ethanol, desalted, and phosphorylized as described for Figure 4.

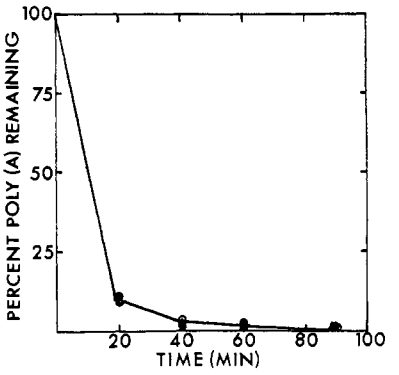


FIGURE 4: Degradation of poly(A) from core-synthesized RNA by polynucleotide phosphorylase. [$^3$H]AMP-labeled RNA (20 Ci/mole) synthesized *in vitro* by cores was mixed with [$^{14}$C]poly(A) (10 Ci/mole) and treated with 100 μg/ml of deoxyribonuclease for 30 min at 37° in a volume of 1 ml containing 10 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$. The reaction mixture was then made 10 mM in EDTA, heated to 95° for 5 min, cooled quickly, and incubated for 30 min with 1 μg/ml of $T_1$ ribonuclease and 10 μg/ml of pancreatic ribonuclease in the presence of 0.3 M NaCl. The resulting poly(A) sequences were extracted with chloroform–phenol (1:1), precipitated with 3 volumes of ethanol, and desalted by passing through a 0.5 × 14 cm column of Sephadex G-25 buffered with 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA. One-half of the poly(A) then was pretreated with 10 μg/ml of alkaline phosphatase for 20 min at 37° in a volume of 0.5 ml containing 0.1 M Tris-HCl (pH 8.3) and 5 mM $MgCl_2$. Phosphorolysis at 37° was initiated by the addition of 10 mM sodium phosphate and 0.6 unit/ml of polynucleotide phosphorylase. At the times indicated, 0.1-ml samples were withdrawn, precipitated on nitrocellulose filters, and counted. The initial material present and symbols used are: (●), alkaline phosphatase-treated [$^3$H]poly(A), 4654 cpm; (▲), alkaline phosphatase-treated [$^{14}$C]poly(A), 415 cpm; (○), nontreated [$^3$H]poly(A), 5284 cpm; (△), nontreated [$^{14}$C]poly(A), 443 cpm.

of each sample was pretreated with alkaline phosphatase to remove any 3′-phosphates present. This preincubation increased neither the rate (see Figure 4) nor the extent of the subsequent phosphorolysis.

Additionally, the phosphate present during phosphorolysis is a strong inhibitor of phosphatases (Torriani, 1968). Thus the phosphorylase does not contain significant levels of phosphatase contamination.

A second problem is that the ribonuclease present during the isolation of the poly(A) might cleave a few of the poly(A) sequences, producing 3′-phosphates. To control this, we added heterologously labeled poly(A) to the RNA before ribonuclease treatment. Any degradation of the endogenous poly(A) would be reflected in degradation of the exogenous poly(A). However, the exogenous poly(A) was always completely susceptible to the phosphorylase, indicating that very little of the poly(A) was degraded by RNase.

Since the polynucleotide phosphorylase contained no phosphatase, and the poly(A) was not being degraded during isolation, then the proportion of poly(A) sensitive to the phosphorylase will reflect accurately the proportion of poly(A) on the 3′ termini of RNA molecules. Figure 4 depicts the results of the incubation of polynucleotide phosphorylase with [$^3$H]poly(A) isolated from RNA synthesized *in vitro* by cores. Over 99% of the poly(A) was rapidly degraded by the phosphorylase. Preincubation of the poly(A) with the phosphorylase enhanced neither the rate nor extent of phosphorolysis, indicating that there were no 3′-phosphates present. The exogenous [$^{14}$C]poly(A) was degraded at the same rate as the endogenous [$^3$H]poly(A). This demonstrates that virtually all of the poly(A) is from the 3′ terminus of mRNA.

Table I lists the results of similar experiments with vaccinia poly(A) synthesized "early" and "late" after infection (before and after the start of viral DNA synthesis, respectively) and with HeLa heterogeneous nuclear and polysomal poly(A). The poly(A) was 95–100% sensitive to the phosphorylase, indicating that virtually all viral and cell poly(A) sequences are on the 3′ termini of their respective RNA molecules.

## Discussion

The above results indicate that a great majority, probably all, of the poly(A) sequences on RNA are on the 3′ termini of nuclear and mRNA molecules. The insensitivity of a small fraction of ribonuclease-resistant poly(A) molecules to either nuclear 3′-exoribonuclease or polynucleotide phosphorylase degradation is not likely to be due to the presence of 3′-phosphates, since preincubation with alkaline phosphatase did not reduce the level of resistance to either exonucleolysis or phosphorolysis. Possibly the resistant RNA was in a double-stranded structure.

Molloy *et al.* (1972) reported that HeLa polyribosomal poly(A), when isolated by ribonuclease digestion in 0.1 M NaCl, is only 73% sensitive to nuclear 3′-exoribonuclease, whereas the HeLa poly(A) isolated by ribonuclease digestion in 0.3 M NaCl in this laboratory is at least 97.6% sensitive to polynucleotide phosphorylase. The higher salt concentration affords better protection against internal "nicking" by ribonuclease (Beers, 1960) and is probably the cause of the disparity between these results and theirs.

This finding that both HeLa nuclear and cytoplasmic poly(A) and L cell nuclear poly(A) are on the 3′ termini of their

respective RNAs is in agreement with the proposal by Darnell *et al.* (1971a) and Philipson *et al.* (1971) that poly(A) is added posttranscriptionally to nRNA. It is not known definitely whether all nuclear poly(A) eventually reaches the cytoplasm, nor whether it is on all heterogeneous nRNA. That poly(A) is on the 3′ end of both mRNA and nRNA does suggest that nRNA is processed such that the 3′ fragment is destined to be mRNA.

Over 90% of the mRNA of vaccinia, which replicates entirely in the cytoplasm, contains poly(A). If poly(A) had a purely nuclear function one would not expect vaccinia RNA to contain poly(A). Thus it is likely that at least one function of poly(A) is cytoplasmic. Whether this concerns the binding of mRNA to ribosomes, the regulation of mRNA stability, or some other function remains to be seen.

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