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Detection of Polyadenylic Acid Sequences in Viral and Eukaryotic RNA

(poly(U)-cellulose columns/poly(U) filters/fiberglass/HeLa cells/bacteriophage T4)

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ABSTRACT **A rapid and specific technique to detect polyriboadenylic acid sequences in RNA is described. The method depends upon the ability of RNAs that contain poly(A) sequences to associate specifically with poly(U) that has been immobilized on fiberglass filters by ultraviolet irradiation. A high proportion of the transcripts synthesized *in vivo* and *in vitro* from the vaccinia virus genome contain poly(A) sequences and bind to the poly-(U) filters. Similarly, DNA-like RNA from the nucleus and from the cytoplasmic polyribosomes of HeLa cells is rich in species that bind to poly(U) filters. Poly(U) immobilized on cellulose powder is useful to make columns with a high capacity for the binding and purification of poly(A)-containing RNAs.**

The occurrence of polyriboadenylic acid and of an enzyme capable of synthesizing this polymer in eukaryotic cells has been known for years (1–10). In general, poly(A) isolated from several animal cells had a base composition of greater than 85% adenine and was about 200 nucleotides in length. Due to the experimental approach used in these earlier studies, it was concluded that the poly(A) existed as a free polymer in the cytoplasm and nucleus of the cells examined. New insight into the poly(A) question was gained by studies with vaccinia virus (11, 12) in which it was demonstrated that poly(A) that averaged 100 nucleotides in length was covalently attached, probably to the 3′-end of the viral RNA. This finding led immediately to the discovery that polysomal RNA extracted from HeLa cells contained sequences of poly(A) (12). An adenosine-rich sequence was also reported in association with globin RNA isolated from rabbit reticulocytes (13). The association of poly(A) sequences with informational RNA has raised important questions concerning the function of this homopolymeric element. Of central importance in a study of the function of poly(A) is the availability of techniques for rapid and specific detection, quantitation, and isolation of poly(A) sequences and of RNAs that contain poly(A) sequences. The basic methods used by researchers in this field depended upon: (*i*) The resistance of poly(A) to digestion by pancreatic and T_1 ribonucleases (7, 12, 14); and (*ii*) The ability of poly(A) to form stable duplexes with immobilized poly(dT) (7, 15) and poly(U) (12, 16).

In this communication, we present a very simple, rapid, and highly specific technique for the quantitation of poly(A) and of RNAs that contain poly(A). The main advantage of this technique, which uses fiberglass filters impregnated with poly(U), is the rapidity with which many samples can be screened for poly(A) content. In this report, we compare the properties of the rapid filter assay with those of the more time-consuming poly(U)–cellulose column assay (12).

MATERIALS AND METHODS

Chemicals. Poly(A), [^{3}H]poly(A), poly(G), [^{3}H]poly(G), and poly(C) were obtained from Miles Laboratories. Poly(U) was obtained from either Miles Laboratories or Sigma Chemical Co. Pancreatic ribonuclease and T_1 ribonuclease were purchased from Worthington Biochemical Corp. Actinomycin D was a gift from Merck, Sharpe and Dohme.

Preparation of Virus and Viral Cores. The WR strain of vaccinia virus was grown in HeLa S_3 cells in spinner culture (17), and virus was purified by a modification (11) of the method of Joklik (18).

Viral cores were prepared from purified virus as described (11), except that Triton X-100 (Sigma Chemical Co.) was used instead of Nonidet P-40 to remove the viral envelope.

Conditions for RNA and poly(A) synthesis *in vitro* by cores, and the purification of RNA were described (11). RNA was stored in 0.01 M Tris·HCl (pH 7.5) at −20°C.

Preparation of HeLa RNA. HeLa S_3 cells (6×10^5 cells/ml) in growth medium were labeled with 4 μCi/ml of [^{3}H]uridine (New England Nuclear) or [^{3}H]adenosine (Schwarz). Nuclear and cytoplasmic fractions were prepared by the method of Penman (19). Polyribosomes were prepared by treatment of the cytoplasmic fraction with 0.25% deoxycholate and centrifuging it through a 5–20% (w/v) sucrose gradient in reticulocyte standard buffer [10 mM Tris·HCl (pH 7.4)–10 mM NaCl–1–5 mM $MgCl_2$] for 5 hr at 5°C at 27,000 rpm in the SW 27 rotor of the Spinco ultracentrifuge. RNA was prepared as described by Kates and McAuslan (20).

Preparation and Use of Poly(U)–Cellulose Columns. About 150 g of Munktell cellulose powder was washed successively with 2 liters of 95% ethanol, 1 liter of 1 N HCl, 5 liters of distilled water, and 1 liter of 95% ethanol, and was then dried at 37°C. To each gram (about 2.5 ml) of washed cellulose powder was added 1 ml of poly(U) solution (8 mg/ml in distilled water). The resultant paste was mixed thoroughly and lyophylized to dryness. Each gram of cellulose in 10 ml of ethanol was poured into a plastic petri dish and irradiated 20 cm from a 30 W Sylvania germicidal lamp for 15 min. The cellulose powder was washed with 50 ml of distilled water, resuspended in 0.01 M Tris·HCl (pH 7.5)–0.1 M NaCl. Usually 0.3–0.4 mg of poly(U) were retained per gram of cellulose. A 0.8 × 20 cm column of poly(U)–cellulose could quantitatively retain more than 1.5 mg poly(A).

RNA dissolved in 0.01 M Tris·HCl (pH 7.5)–0.1 M NaCl was loaded on a poly(U)–cellulose column equilibrated at 4°C with the same buffer. The column was washed for 30

min with 15 ml of 0.01 M Tris·HCl (pH 7.5)-0.1 M NaCl at 4°C, and again at 25°C with 0.01 M Tris·HCl (pH 7.5).

Preparation and Use of Poly(U)–Fiberglass Filters. To each fiberglass filter (Whatman GF/C, 2.4 cm diameter) supported on a rubber grid was added 0.15 ml of poly(U) solution (1 mg/ml in distilled water). The filters were then dried at 37°C and irradiated for 2.5 min on each side at a distance of 22 cm from a 30 W Sylvania germicidal lamp. Each filter was rinsed with 50 ml of distilled water to remove unbound poly(U). About 67% of the input poly(U) was retained per filter. 10-times more poly(U) is retained on the filter after irradiation if the amount of RNA added is increased 10 times.

Radioactive RNA to be tested for binding to poly(U) was dissolved in 0.5–10 ml of binding buffer (0.01 M Tris·HCl (pH 7.5)–0.12 M NaCl), then filtered at 2 ml/min through poly(U)–fiberglass filters that had been equilibrated with the the same buffer at 25°C. The filters were washed with 20 ml of binding buffer, followed by 20 ml of ice-cold 5% trichloroacetic acid and 10 ml of 95% ethanol. The filters were dried and counted.

Ribonuclease Assay for Poly(A). Ribonuclease resistance of RNA was determined by digestion of the RNA with 10 μg/ml of pancreatic ribonuclease and 1 μg/ml of T_1 ribonuclease in 0.01 M Tris·HCl (pH 7.5)–0.2 M KCl in the presence of 0.1 mg/ml of unlabeled poly(A) for 20 min at 37°C.

TABLE 1. *Retention of RNA by immobilized poly(U), a comparison of filters and columns**

RNA	Percent retained on poly(U) column	Percent retained on poly(U) filter	Percent resistant to ribonuclease
1. [^{3}H]Poly(A)	100	100	100
2. [^{3}H]Poly(G)	—	<1†	—
3. [^{14}C]UMP-labeled *E. coli* RNA	—	0	—
4. [^{3}H]UMP-labeled T4 late RNA	0.47*	—	0.5*
[^{3}H]AMP-labeled T4 late RNA	0.74*	<0.1	—
[^{3}H]AMP-labeled T4 late RNA mixed with 10 μg/ml of poly(A)	—	<0.1	—
5. [^{14}C]AMP-labeled core RNA	55*	85	27–30*
[^{3}H]UMP-labeled core RNA	35*	65	<1*
6. [^{14}C]AMP-labeled core RNA bound to poly(U) cellulose on previous fractionation	95*	—	50–60*
[^{14}C]AMP-labeled core RNA not bound to poly(U) cellulose on previous fractionation	<1*	—	<1*
Vaccinia RNA synthesized *in vivo* by cores 1 hr after infection			
7. [^{3}H]UMP-labeled cytoplasmic RNA	36*	—	—
[^{3}H]AMP-labeled cytoplasmic RNA	—	—	16–20
Total cytoplasmic RNA at 4.5 hr			
8. [^{3}H]UMP-labeled RNA	53*	68	—
[^{3}H]AMP-labeled RNA	56*	—	20*
9. Polyribosomal RNA at 4.5 hr			
[^{3}H]UMP-labeled RNA	42*	47	—
[^{3}H]AMP-labeled RNA	44*	—	11

Late RNA (5–8 min) was prepared from T4 phage (20). RNA synthesized *in vitro* by vaccinia cores (11) was labeled simultaneously with [^{14}C]ATP and [^{3}H]UTP. RNA was labeled *in vivo* with radioactive nucleoside for 10 min either 1 hr after infection in cells treated with streptovitacin A (15 μg/ml) from the time of virus addition, or 4.5 hr after infection (20). RNA from cytoplasmic and polyribosomal fractions was prepared as described in *Methods*. Purified RNA was either fractionated on a poly(U)–cellulose column, bound to a poly(U)–fiberglass filter, or assayed for ribonuclease resistance.

* Data reprinted from Kates, J. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 743–752.

† Corrected for a background of 4% on a filter that did not contain poly(U).

RESULTS

Binding of various RNAs to immobilized poly(U)

To determine the specificity of binding of RNAs to poly(U)–fiberglass filters, various RNA samples were applied and the fraction remaining bound to the filter was determined. Since poly(U)–cellulose columns were used routinely in our laboratory before the adoption of the filter method (11), it was also of interest to compare the results obtained on the poly(U) columns with those obtained by the filter technique, particularly in view of the fact that the RNA is in contact with the poly(U) filter for only a small fraction of the time required to percolate the RNA through the poly(U) columns. Some of the data in Table 1 is taken from a previous publication (11). The poly(A) synthesized *in vitro* by vaccinia virus cores is 200 nucleotides long and contains 100% adenine; it was bound quantitatively to both the column and the filter.

Poly(G), *Escherichia coli* rRNA, and T4 RNA labeled between 5 and 8 min after infection were not retained. The failure of T4 RNA to bind to poly(U) suggests that this RNA lacks poly(A) sequences. In fact, Table 1 shows that less than 1.5% of [^{3}H]AMP-labeled RNA from T4 phage was resistant to RNase digestion—a test for poly(A) content. Simple addition of unlabeled poly(A) to radioactive RNA from T4 phage did not result in any binding of T4 RNA to poly(U), indicating that some readily formed association between mRNA and poly(A) is not responsible for the retention of mRNA on poly(U) filters.

In contrast to T4 phage RNA, transcripts from the vaccinia virus genome bind well to immobilized poly(U). Core RNAs synthesized *in vitro* and labeled with radioactive ATP or UTP were bound 55–85% and 35–65%, respectively, to immobilized poly (U). The higher binding in the ranges indicated above was always to the poly(U)–fiberglass filter, and the lower to the poly(U)–cellulose column. The retention of vaccinia virus RNA on poly(U) filters implies that this RNA contains attached poly(A) sequences. It may be seen in Table 1 that 27–30% of the [^{14}C]AMP-labeled core RNA was resistant to RNase digestion. If the poly(A) moiety is responsible for the binding of core RNA to immobilized

poly(U), then all of the RNase-resistant RNA labeled with [^{14}C]AMP should be retained by poly(U), and none should occur in the fraction of RNA that is not retained. As seen in Table 1, all of the RNase-resistant [^{14}C]AMP (50–60% of the total AMP bound) was retained by a poly(U) column. Furthermore, nearly all of the core RNA that was previously retained by a poly(U) column was bound to a subsequent poly-(U) column, and the fraction of core RNA that was previously excluded was also excluded on a subsequent poly(U) column fractionation. Since about equal (molar) percentages of adenine and uracil occur in vaccinia core RNA (unpublished observation), it is surprising that about 50–60% of the [^{14}C]AMP radioactivity in core RNA that bound to poly(U) is resistant to RNase. This finding indicates that the non-poly(A) moiety of this RNA is low in adenine content.

Vaccinia RNA purified from cytoplasm of infected cells and polyribosomes either 1 hr or 4.5 hr after infection also bound extensively to immobilized poly(U).

Binding of HeLa cell RNAs to poly(U) filters

HeLa cells were labeled with radioactive adenosine and/or uridine for 30 min. RNA was purified from the nucleoplasm and from isolated polyribosomes. Cells were also labeled for 24 hr with the same precursors, and incubated with unlabeled adenosine or uridine for an additional 12 hr (Table 2). Under the latter conditions, more than 95% of the cytoplasmic radioactivity occurs in ribosomal and transfer RNAs (21). Little, if any, of the stable RNA species bound to poly(U), although very substantial amounts of RNA labeled in short-term incubations were retained. Polysomal mRNA is very rich in poly(A), since about 50% of the mRNA binds to poly(U) filters, and since about 15% of the AMP radioactivity is resistant to RNase digestion. Nuclear RNA contains lower relative amounts of poly(A) than does polysomal mRNA. A more thorough analysis of polypurine sequences in HeLa cell RNA and their significance will be presented elsewhere (Jurale and Kates, in preparation).

TABLE 2. *Retention of HeLa RNA by poly(U)-fiberglass filters*

RNA	Percent retained on poly(U) filter	Percent resistant to ribonuclease and binding to poly(U) filter
30-min label		
1. [^{3}H]AMP-labeled nucleoplasmic RNA	12.5–20	1.53
[^{3}H]UMP-labeled nucleoplasmic RNA	9.5–18	0
2. [^{3}H]AMP-labeled poly-rRNA	45–60	10–15
[^{3}H]UMP-labeled poly-rRNA	45–50	0
36-hr Label		
3. [^{3}H]UMP-labeled rRNA	1.6	—
[^{3}H]AMP-labeled rRNA	—	0

HeLa RNA was labeled for either 30 min or 36 hr and purified. After 36 hr of labeling, nearly 100% of the radioactivity in the polyribosomal RNA occurs in 18S and 28S rRNA (21). The RNA was assayed for ribonuclease resistance and for its ability to bind to poly(U) filters.

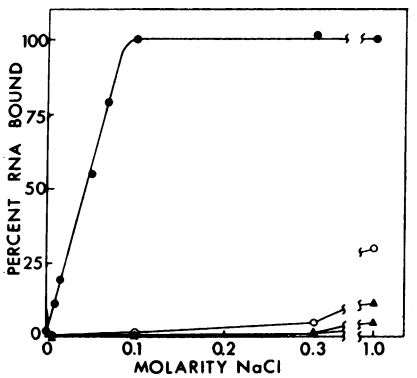

FIG. 1. Salt dependence of the retention of RNA by fiberglass filters. [^{3}H]Poly(A) (*circles*) and [^{3}H]AMP-labeled late RNA from T4 phage (*triangles*) in solutions of NaCl of the indicated molarity buffered with 0.01 M Tris·HCl (pH 7.5) were bound to fiberglass filters, some of which contained poly(U) (*closed symbols*), the rest of which did not (*open symbols*).

Characteristics of RNA binding to poly(U) filters

If the binding of RNA to immobolized poly(U) is due to poly(A)·poly(U) duplex formation, then the binding should be dependent on the concentration of cations (22). Fig. 1 shows that the fraction of poly(A) retained by the poly(U) filters increased linearly with NaCl concentration, up to 0.1 M where retention was complete. Retention of poly(A) by filters lacking poly(U), (i.e., blank filters), was significant only at salt concentrations above 0.1 M.

If the binding of poly(A) and other RNAs to poly(U) is through complementary base pairing, then this binding should be specifically inhibited by prior incubation of the RNA with poly(U) in solution. Table 3 demonstrates that poly(C), poly(G), and rRNA do not prevent the binding of poly(A), but that similar concentrations of poly(U) completely abolished binding. High concentrations of poly(G) significantly decreased the amount of poly(A) retained. This result, however, can be explained on the basis of the known ability of poly(G) to form complexes with poly(U) (22). Prior incubation of poly(U) with core RNA also abolishes the ability of core RNA to bind to poly(U) filters.

TABLE 3. *Effect of RNA on the retention of poly(A)*

RNA	Amount of competing RNA (μg)	Percent labeled RNA retained on filter
[^{3}H]poly(A)	—	100
[^{3}H]poly(A) and poly(C)	10	96.5
	100	100
[^{3}H]poly(A) and poly(G)	10	104
	100	41
[^{3}H]poly(A) and *E. coli* rRNA	about 100	104
[^{3}H]poly(A) and poly(U)	10	<1
[^{3}H]UMP-labeled core RNA	—	65
[^{3}H]labeled core RNA and poly(U)	10	<1

[^{3}H]Poly(A) (25 ng) and [^{3}H]UMP-labeled core RNA (10 ng) were incubated for 10 min in 1 ml of binding buffer [0.01 M Tris·HCl (pH 7.5)–0.12 M NaCl] at 25°C, then bound to poly-(U) filters.

The melting point of poly(A)·poly(U) duplexes in 0.1 M NaCl is about 57°C (20). It was, therefore, of interest to compare the temperature dependence of release of poly(A) and RNA from poly(U) filters with the known melting temperature (Tm) for poly(A)·poly(U). In fact, Fig. 2 demonstrates a sharp temperature transition for the release of poly(A), with a Tm of 57°C. Core RNA labeled with either UMP or AMP was released over a broader temperature range. The breadth of the core RNA transition may reflect the influence of the moiety other than poly(A) on the stability of the complex with the filter. If AMP-labeled core RNA is digested with T1 RNase and then applied to a poly(U) filter, the melting point of the resulting complex is similar to that of poly(A)·poly(U).

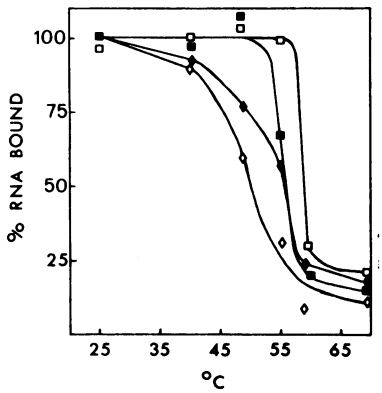

FIG. 2. Temperature-dependent release of RNA from poly(U)-fiberglass filters. 42 μg of vaccinia core RNA labeled with [^{3}H]AMP was digested with 7 μg of T1 ribonuclease in 1.0 ml of 0.01 M Tris·HCl (pH 7.5)–0.01 M EDTA for 60 min at 37°C. The T1 ribonuclease-resistant RNA (■——■), core-synthesized [^{14}C]poly(A) (□——□), [^{3}H]UMP-labeled core RNA (◇——◇), and [^{3}H]AMP-labeled core RNA (◆——◆) were bound to poly(U)-fiberglass filters. The RNA–filter complexes were incubated in binding buffer at the specified temperatures for 10 min. The radioactivity retained on the filters after the incubation is expressed as percent of the RNA bound at 25°C.

Capacity of poly(U) filters

Theoretically, the fraction of input poly(A) that is retained by the poly(U) filter should increase with the time allowed for annealing. Since in our assay with poly(U) filters the application of sample is relatively rapid (2 ml/min), it was critically important to determine an upper limit to the amount of poly(A) that could be applied and quantitatively retained under these conditions. Therefore, a small fixed amount of radioactive poly(A) was mixed and applied to filters containing poly(U). Inputs of up to 1 μg of poly(A) were retained completely. However, only 6–7 μg of poly(A) were bound when 10 μg of poly(A) was applied.

DISCUSSION

In this report we have described a rapid and simple method for the detection and isolation of RNAs that contain poly(A) sequences. This technique is based on the ability of poly(A)-containing RNAs to form specific complexes with immobilized poly(U). Poly(U)–cellulose columns are primarily useful for preparation of moderate quantities of poly(A)-containing RNA, but are too time-consuming to be used for routine assays of poly(A) sequences in RNA samples. Poly(U)–fiberglass filter discs, on the other hand, are well suited for rapid assays of RNA species containing poly(A).

Several characteristics of RNA binding to poly(U) filters indicate that poly(A)·poly(U) duplex formation is the principle involved: (*i*) The elution of poly(A) from poly(U) filters shows a sharp temperature-dependent transition, with a midpoint close to the Tm observed for poly(A)·poly(U) duplexes in a solution of similar ionic strength (20); (*ii*) The binding of RNA to poly(U) filters is prevented if the RNA is first incubated with poly(U) in solution; (*iii*) Only RNAs that contain RNase-resistant poly(A) sequences bind to the filters; and (*iv*) The binding of RNA to poly(U) filters shows a dependence on cation concentration that is characteristic of duplex formation (21).

The experimental results presented, coupled with our previous study on the poly(A) content of vaccinia RNA (11, 12), indicate that poly(A) sequences about 100 nucleotides long are attached covalently at the 3′-end of high molecular weight, DNA-like RNA. This attachment is most readily shown by the binding of UMP-labeled RNA to immobilized poly(U). The RNA that binds to poly(U) has a size distribution characteristic of vaccinia polyribosomal RNA and hybridizes readily with vaccinia DNA (12). The mode of synthesis of the poly(A) sequences in vaccinia RNA remains uncertain, although several lines of evidence favor a DNA template mechanism, at least in part (11, 12).

In addition to our previous findings of poly(A) in the polysomal mRNA fraction of HeLa cells (12), in this report we demonstrate the occurrence of poly(A) sequences in the large heterogeneous nuclear RNA of HeLa cells. Though the implications of this finding will be discussed in detail elsewhere (Jurale and Kates in preparation), the occurrence of poly(A) sequences in nuclear RNA are indicative of a possible precursor–product relationship between a portion of the nuclear RNA species and the cytoplasmic mRNA (23).

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Addendum. Since the original writing of this manuscript, several laboratories have reported the occurrence of poly(A) sequences attached to HeLa and mouse ascites cell RNA (23–25). Two of these reports confirm our findings of poly(A) sequences in HeLa polyribosomal RNA and, in addition, one of the reports (23) demonstrates the association of poly(A) with the heterogeneous nuclear RNA. Also of interest is the report of a nitrocellulose filter-binding method for the detection and quantitation of poly(A) sequences and of RNAs containing poly(A) (25). It should be noted that the nitrocellulose filter technique possesses many of the virtures of the poly(U)–filter method described in this report. Since the basis of binding of an RNA to a nitrocellulose filter is unknown, use of poly(U) filters in conjunction with the nitrocellulose filters might be a useful check, especially when new types of RNA are tested.

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