Preparation and properties of a poly A resin and its use in the isolation of naturally occurring poly U sequences

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ABSTRACT

A method for the covalent attachment of poly A, as well as other nucleic acids and nucleosides, to a methylene dianiline derivative of starch is described. The properties of this poly A resin and its use for the recovery of poly U sequences from both nuclear and cytoplasmic extracts of HeLa cells is described.

INTRODUCTION

Poly U sequences of 30 to 40 nucleotides have been isolated from ribonuclease T₁ digests of the heterogeneous nuclear RNA (HnRNA) of HeLa cells on poly A sepharose¹ and on poly A trapped in polyacrylamide gels². Significant amounts of polyuridyate sequences have also been detected in the polysomal RNA of HeLa cells with the latter technique^{1,2}. Although similar approaches have been used, the isolation of poly U sequences has presented certain problems which were not encountered in the isolation of poly A sequences. One technical problem stems from the use of affinity materials in which the poly A used to bind poly U was not covalently attached. This is the case for the poly A trapped in polyacrylamide gels² and also may be true of poly A bound to Sepharose 4B by a commonly used method⁴. In our hands, each of these materials showed a slow release of poly A from the solid support which not only limited their re-use, but more importantly, variable amounts of poly A may be released along with the poly U that is removed at low ionic strength. A more fundamental problem is created by the molar excess (5-10 fold) of the much longer poly A sequences in HeLa cell RNAs. This is an unfavorable situation for the isolation of poly U sequences since they tend to hybridize to these poly A sequences, particularly if intramolecular, rather than to a poly A affinity column.

In this report we describe a method for the covalent binding of poly A as well as other nucleic acids and nucleosides to a methylene dianiline derivative of starch by azo coupling. Some properties of this poly A

resin¹⁴ and the conditions which permit maximal recovery of poly U sequences from RNA preparations containing an excess of poly A sequences are described.

MATERIALS

³H-Adenosine (3.1×10^5 cpm/ μ mole), ³H-ATP (2.4×10^5 cpm/ μ mole) and ³H-poly A (4.5×10^5 cpm/mg) were prepared by appropriate dilution of preparations from New England Nuclear and from Schwarz/Mann in the case of ³H-poly A. ³²P-Poly A was prepared from HeLa cell nuclear RNA.⁵ *E. coli* tRNA and ovalbumin were from Sigma Biochemicals; dialdehyde starch, a periodate oxidized product of starch (Sumstar 190) from the Sumner Corporation, subsidiary of Miles Laboratory, Elkhart, Ind.; DNase I (DPFF) was from Worthington Biochemicals.

METHODS

Cell Labeling, Fractionation and RNA Isolation. Maintenance, ³²P-labeling and fractionation of HeLa cells have been described, as have techniques for RNA and poly A isolation^{5,13}.

Isolation of Poly U Sequences from RNA. RNA (250 to 500 μ g) was digested with 200 units of ribonuclease T₁ in 1.7 ml of 0.03M Tris HCl, pH 7.4 at 37° for 15 min. The reaction was chilled to 0° and CaCl₂ and MgCl₂ were added to give final concentrations of 2 and 8 mM respectively. Pancreatic DNase I (10 μ g) in 0.1M sodium acetate buffer, pH 6.0 was added and incubation continued for 15 min at 37°C. Reactions were stopped by adding SDS and EDTA to final concentrations of 0.5% and 0.05M respectively. Unlabeled commercial poly U (30 μ g), was then added to each sample followed by NaCl to a final concentration of 0.1M before 10 mg of poly A resin suspended in 1 ml of NETS was added. After the mixtures were shaken for 30 min at 23°, the resin recovered on a coarse sintered glass funnel was washed with 10 x 3 ml portions of 0.1M NaCl, 0.01M EDTA, 0.01M Tris pH 7.2, 0.5% SDS (NETS) at 23°. Poly U was eluted from the resin with 6 x 1.0 ml portions of ETS (NETS without NaCl) buffer preheated to 60°C. The eluates were pooled and aliquots were taken to measure TCA precipitable radioactivity. The remainder was precipitated with ethanol after adding 100 μ g of yeast RNA.

Purification of Poly U Sequences. The poly U obtained from poly A containing RNA preparations was invariably contaminated with adenylate-rich sequences which were removed by electrophoresis on 10% polyacrylamide gels. Figure 3 shows poly U as a distinct peak, moving more slowly than the tracking dye, but more rapidly than a broad adenylate-rich component. Eluates

pooled from fractions of both components as indicated were filtered through sintered glass funnels to remove coarse gel particles. Each filtrate was then applied to a small Sephadex A25 column (0.5 x 0.5 cm) equilibrated with 0.02M sodium acetate buffer (pH 5.5) containing 7M urea. The columns were washed with 5 x 1.0 ml portions of this buffer after the samples were loaded. Poly U was eluted with 4 x 0.5 ml portions of 0.02M sodium acetate buffer pH 5.5 containing 7M urea and 1.8M NaCl. Poly A was eluted with 4 x 0.5 ml of 1.4M sodium phosphate buffer pH 5.5 containing 7M urea. After the addition of 200 μ g of yeast RNA, poly U was precipitated from the eluates with ethanol. Poly A samples were diluted 30-fold to reduce the phosphate concentration before adding 500 μ g of yeast RNA and 2 volumes of ethanol. In the latter case after 18 hrs at -20° , a flocculent precipitate of sodium phosphate appears which can be dissolved by the careful addition of 1M HCl with shaking to maintain the pH at 5.0 or slightly higher. Poly A can then be quantitatively recovered by centrifugation of the ethanol suspension.

Base Composition Analysis. RNA precipitates recovered from ethanol and dried with ether were digested with 15 μ l of 0.33M KOH for 18-20 hrs at 37° . KOH was neutralized with HClO₄ and the insoluble KClO₄ was removed by centrifugation at 4° C. The supernatants were analyzed for base composition by high voltage paper electrophoresis as described.¹⁵

Acid Precipitation of Poly U. Because poly U, in contrast to RNA or poly A tends to be soluble in acid⁶, the following procedure was developed to ensure its complete precipitation. Aliquots of poly U (10-15 μ l) were diluted into 0.5 ml of NETS before the addition of 30 μ g of poly A and 200 μ g of bovine serum albumin. The mixtures kept at 4° C for 10 minutes were then precipitated with an equal volume of 20% TCA. After 10 minutes in ice the precipitates were collected on glass fiber filters (Whatman GF/A) and were washed twice with 5% TCA, dried and counted directly for Cerenkov radiation.

RESULTS

Preparation and Properties of S-MDA Bound Nucleotides and Polynucleotides. Amino groups in general react easily with diazonium salts under mild conditions. Enzymes have been coupled to polydiazonium salts on inert matrices with substantial retention of activity⁷⁻¹⁰. Goldstein, *et al.* reported preparation of a novel diazotizable starch derivative to which enzymes could be coupled with substantial retention of activity¹⁰. The material is prepared by condensing dialdehyde starch (DAS), a periodate

oxidation product of starch with p,p' diamino diphenylmethane (methylene dianiline or MDA). The Schiff base formed is then reduced to a secondary amine with sodium borohydride. By adjusting the ratio of MDA to DAS, a polymeric product (S-MDA resin) with minimal cross linking and maximal diazotization capacity can be prepared. In preparations used here 10 gm of DAS and 30 gm of MDA were reacted in 300 ml of methanol. After preparation the resin was washed extensively with methanol, dried overnight *in vacuo* over anhydrous CaCl_2 . It has been stored in the dark at room temperature for over 6 months with no apparent loss of activity.

Coupling of Nucleotides and Nucleic Acids to S-MDA Resin. S-MDA resin (0.15 gm) was diazotized as described⁹ and the diazotized cake was washed with 25 ml of potassium phosphate buffer (0.2M, pH 7.8) in a Buchner funnel before suspension in 10 ml of the same buffer containing ^3H -adenosine or ^3H -ATP as described in Table 1. The slurry was shaken in a wrist action

TABLE 1: Attachment of Compounds to Methylendianiline Starch by Diazotization.

REACTANT	AMOUNTS REACTED	AMOUNT BOUND*	AMOUNT BOUND†
		%	
^3H -Adenosine	14 μmoles	63.4	--
^3H -ATP	16 μmoles	< 1.0	--
^3H -Poly A	2.5 mg	82.5	--
^{32}P -Poly A	2.5 mg	--	71.0
tRNA [‡] (<i>E. coli</i>)	21.0 mg	26.0	30.6
Ovalbumin	30.0 mg	90.0	--

*Measured as difference between the amount added and amount not bound divided by the total amount added.

†Measured as amount of bound product recovered from resin after alkaline hydrolysis (see Methods).

‡1.5 gm of S-MDA resin was used in this experiment instead of 0.15 gm.

shaker for 18-24 hours. It was then filtered through a coarse sintered glass funnel and the filtrate was analyzed for radioactivity. The resin was washed twice with 10 ml of the above buffer and twice with 10 ml of 0.02M sodium phosphate buffer (pH 5.5) containing 7M urea and 1.8M NaCl.

After coupling either poly A or RNA to the resin, the product was washed successively with 2 x 10 ml of 0.2M potassium phosphate buffer (pH 7.8), 2 x 10 ml of 1.4M sodium phosphate buffer (pH 5.5) containing 7M urea and 2 x 10 ml of 98% aqueous formamide. The amount of covalently bound ligand was determined either as the difference between the input and the unbound radioactivity (filtrate plus washes) or, in the case of unlabeled ligands, as the difference in absorbance units at 260 nm. In one case

nucleic acid coupled to resin was actually released with 0.33M KOH at 37° for 18 hours. The hydrolysate was neutralized before insoluble material was removed. An aliquot of the solution was analyzed for alkali-labile radioactivity and absorbance at 260 nm (Table 1).

For larger preparations, 50 mg commercial poly A was coupled either to 1 or 2 gm of S-MDA resin. Efficiency of incorporation was tested in a small reaction in which ³²P labeled natural poly A was coupled to 0.15 gm S-MDA resin (Table 1). After washing as above, poly A resin was dehydrated by acetone washing, dried and stored in the dark at 4°C. Alternatively, it can be stored as a suspension at 4° in NETS.

Coupling data for selected biochemicals are given in Table 1. ATP could not be coupled, but adenosine was efficiently bound. Because aromatic amino groups are especially reactive in azo coupling reactions the 6-amino group of adenine would be expected to be the major reactive group. The non-reactivity of ATP may be due to the shielding effect of the phosphate on the ring amino nitrogen as was suggested by Sundaram who also found that adenosine, but not ATP, could be coupled to Sepharose activated by CNBr or mixed carbonic anhydrides¹¹. Poly A was efficiently coupled as indicated by the fact that in two separate preparations more than 70% of the label was bound. Transfer RNA can also be bound while proteins such as ovalbumin are bound very efficiently under the same conditions.

Properties of Poly A Resin. The covalent attachment of poly A to the S-MDA resin is indicated by the fact that extensive washing with buffers containing either 7M urea and 1.4M sodium phosphate (pH 5.5) or 98% formamide did not release poly A. Poly A was released from the resin only after prolonged alkaline hydrolysis (Table 1). In some preparations as much as 40 mg of poly A was coupled to 1 gm of dry S-MDA resin. Poly A resin can be dehydrated by acetone washing, dried and stored indefinitely in the dark at 4°. Before use, weighed amounts can be suspended in the buffer by gently breaking the clumps with a glass rod. The fines are removed by filtering the suspension through a sintered glass funnel. By avoiding excessive stirring during preparation, a material with excellent flow rates suitable for column use is obtained. Storage of extensively re-utilized poly A resin in NETS at 4° resulted in little loss of poly U binding capacity over a 6-month period. After each use, the resin was washed with dilute buffers containing 7M urea followed by 98% formamide.

Hybridization Efficiency. Figure 1 illustrates the binding curves for poly U obtained with two different batches of poly A resin. The resin with

Figure 1: Hybridization Capacity of Poly A Resin. ^3H -Labeled poly U (20,000 cpm; 0.75 μg) was mixed with fixed amounts of unlabeled commercial poly U in 1.0 ml NETS. Ten mg of poly A resin suspended in 0.5 ml of NETS was added to each poly U solution. The tubes were vigorously shaken for 30 minutes at 23°C after which the resin was filtered through a coarse sintered glass funnel. The funnel was washed ten times with 0.5 ml NETS at 23°C. The pooled washes are the unbound fraction. Poly U was eluted with 5 successive 0.5 ml portions of NETS preheated to 60°. Radioactivity in 0.5 ml aliquots of the unbound fractions and of the pooled eluates was measured in 10 ml PCS, a liquid scintillorfluor from Amersham Searle Corp. —□—□, poly A resin containing 40 mg poly A coupled per gm of resin; —Δ—Δ, and —●—● poly A resin containing 20 mg poly A per gm of resin; —○—○, 50 μg poly A mixed with 50 μg ^3H -poly U (20,000 cpm) before binding; —□—□, 50 μg poly A mixed with 10 μg ^3H -poly U (20,000 cpm) before binding.

the highest capacity bound 200 μg poly U per 10 mg of resin. At the highest level of hybridization observed (top curve) about 50% of the poly A sequences of the resin were complexed with poly U. Although azo coupling via the 6-amino group of adenine might be expected to interfere with hydrogen bonding to uracil, use of commercial poly A containing molecules as long as 1000 nucleotides apparently permits the poly A to be coupled to the resin at a limited number of residues. The variation in the binding capacity of the two preparations seen here may reflect differences in this number. Very

Figure 2: Polyacrylamide Gel Electrophoresis of ^3H Poly U Recovered on the Poly A Resin. ^3H -Poly U was added to a nuclear fraction (Table 2). RNA was then extracted, and digested with RNase T₁. The digest was treated with poly A resin as described in the legend to Table 2. Polynucleotides recovered from ethanol were electrophoresed on 10% polyacrylamide gels as described. The gels were fractionated by a Maizel fractionator using 7M urea as solvent. Electrophoresis is from left to right. o—o, unbound fraction; Δ — Δ , bound fraction; $\bullet$ — $\bullet$, unfractionated commercial ^3H -poly U added to extracts.

small amounts of poly U, i.e. $<5\ \mu\text{g}$, are not always quantitatively removed by the conditions described in Figure 2. This may reflect the concentration dependence of the bimolecular annealing process since this can be overcome by carrier addition (Table 3). That poly U binding is due to hybridization to the poly A resin is shown by the fact that an addition of an ex-

cess of unlabeled poly A prevented the binding of poly U (Fig. 1).

Isolation of Poly U Sequences in Cells with Poly A Resin. The quantitative isolation of natural poly U sequences has posed certain problems not encountered in the isolation of poly A sequences using essentially similar techniques. These stem from the fact that in the cell poly U sequences usually co-exist with a molar excess of much longer poly A sequences. Techniques for poly U isolation based on hybridization to poly A must effectively compete with native poly A sequences to be quantitative. To be certain that this can be achieved in HeLa cells, a 4 to 5-fold excess of ^3H -poly U was added both to nuclear and cytoplasmic extracts in one case prior to, and in the other, after RNA isolation before the standard poly U isolations were carried out (Table 2). The recovery of the added poly U in an

TABLE 2: Availability of Exogenous Poly U Sequences in Cell Extracts for Hybridization to Poly A Resin.

^3H -POLY U ADDED TO:	^3H -POLY U RECOVERED
	cpm
1. NETS	60,000*
2. Cytoplasmic extract	51,500
3. Cytoplasmic RNA	54,500
4. Nuclear extract	51,500
5. Nuclear RNA	56,500

*This represents 85% of the ^3H contained in a commercial ^3H -poly U sample.

Hypotonically swollen HeLa cells (1.36×10^8 cells) were homogenized with Dounce homogenizer and separated into nuclear and cytoplasmic fractions⁵. Each fraction was divided into two equal portions. ^3H -Labeled poly U (70,000 cpm; 2.6 μg) was added to one portion of nuclei and one of cytoplasm. RNA extracted from all four fractions⁵ was precipitated with ethanol. The precipitates were dissolved in 1.5 ml H_2O . ^3H -Labeled commercial poly U was then added to the RNA fractions that had not received it previously. Poly U was isolated from each RNA as described in Methods. After determining the total radioactivity in the unbound and bound fractions, 100 μg of yeast RNA was added to each sample before addition of ethanol to precipitate RNA.

essentially undegraded state is nearly quantitative under these conditions (Figure 2). Those sequences that failed to bind to the resin were polydisperse on electrophoresis. Some of the more rapidly migrating unbound sequences may have been too short to form stable hybrids with poly A while those migrating more slowly may be poly U:poly A hybrids formed with poly A sequences released from RNA by RNase T₁. From these data it should not be concluded that all endogenous poly U is recoverable since complexes with poly A in hairpin or looped structures could remain undetected under these conditions.

The need for the addition of exogenous poly U to recover endogenous poly U in high yield from both nuclear and cytoplasmic extracts is stressed

TABLE 3: The Effect of the Addition of Carrier Poly U on the Recovery of Natural Poly U Sequences.

SAMPLE	RNA ANALYZED ³² P	+ POLY U*	+ POLY U [†]	- POLY U
	cpm x 10 ⁶		cpm	
<u>Nucleus</u>				
Total RNA	67.2	73,020	78,466	16,790
Poly A(+) RNA	17.4	50,032	--	8,180
<u>Cytoplasm</u>				
Total RNA	67.4	15,708	12,043	2,700

*Poly U added to extracts prior to RNA isolation.

[†]Poly U added to extracted RNA.

HeLa cells (1.92×10^8 cells) were labeled with 50 mCi ³²P at a concentration of 2×10^6 cells/ml for 4 hrs. Nuclear and cytoplasmic fractions were prepared as described⁵. The first wash of the nuclear pellet was added to the cytoplasmic suspension⁵. The nuclear pellet was suspended in 10 ml 0.05M sodium acetate, pH 5.2 containing EDTA at 0.01M. 30 µg of poly U was added to 1/10th of this nuclear suspension before RNA was isolated from this aliquot and from the remaining 9/10ths of the nuclear suspension by the usual SDS:phenol extraction at 60°. The RNA from the larger fraction was dissolved in 1.5 ml H₂O before two aliquots each equivalent to that of the small aliquot described above were removed and 30 µg of poly U was added to one of them. The remaining RNA solution was treated with oligo dT cellulose to isolate poly A containing HnRNA¹³.

A post-mitochondrial fraction was prepared from the combined cytoplasmic suspension and nuclear wash by centrifugation at $10,000 \times g$ for 15 minutes. Poly U was added to 1/10th of this supernatant before RNA was isolated from this aliquot and from the remaining suspension as described above. The RNA recovered from the larger fraction was resuspended in 1.5 ml of water. Two aliquots, each equivalent to the aliquot previously taken were removed and 30 µg of poly U was added to one. Poly U was purified and quantitated from each of these nuclear and cytoplasmic RNAs as described in Methods, but without the further addition of poly U carrier.

by the data of Table 3. Major losses of poly U sequences were observed in cases where carrier poly U was not added although it made little difference whether it was added prior to or after RNA extraction. This addition, although necessary, does complicate the methodology of quantitating poly U when it is measured in preparations of poly A containing RNA. This is seen most strikingly in the gel electrophoretic patterns of the products bound to the poly A resin (Figure 3). In products bound from both total nuclear (Fig. 3A) and total cytoplasmic RNA (Fig. 3B), two components are invariably separated on gels. The most rapidly migrating (component II) is obviously UMP-rich but a major fraction of radioactivity, particularly from the cytoplasmic RNA, that migrates more slowly and heterogeneously is AMP-rich. The UMP-rich component has been purified further and is an uninterrupted sequence of 30 to 40 UMP's^{2,3}. Since the AMP-rich component invariably contaminates poly U sequences the quantitation of poly U in poly A containing RNA requires this additional gel electrophoretic step after binding

Figure 3: Polyacrylamide Gel Electrophoresis of HeLa Nuclear and Cytoplasmic Poly U Fractions. ^{32}P -labeled fractions prepared from RNase T $_1$ digests of HeLa nuclear and cytoplasmic RNAs and bound to poly A resin as described in Methods were electrophoresed on 10% polyacrylamide gels. The gels were fractionated as described and fractions counted for Cerenkov radiation. The indicated fractions were pooled and processed for base composition as described in the text. Direction of electrophoresis is from left to right. ^{32}P -bound to poly A resin from (A) total nuclear RNA, (B) total cytoplasmic RNA.

to poly A resin.

This tendency for some poly A (usually about 10 percent of the total) to accompany poly U through the isolation is not clearly understood. Since it separates so readily from poly U during electrophoresis (Fig. 3) and on DEAE Sephadex², it is clearly not covalently linked to poly U. Since the relative amount of poly A which accompanies poly U can be reduced by lowering the amount of carrier poly U used, we presume that the excess of carrier poly U required for these experiments will also hybridize to the poly A resin, and thereby carry along some labeled poly A segments that have annealed to other single stranded regions of the poly U carrier.

DISCUSSION

Affinity chromatography of nucleic acids employing polynucleotides coupled to inert matrices is widely used to isolate complementary oligonucleotide stretches from partial digests of nucleic acids and the intact

nucleic acids containing these stretches. An ideal adsorbent of this type should possess these features: (i) the reactive moiety should be covalently attached to the inert matrix at as few points as possible to avoid steric hindrance, (ii) the matrix should be inert and (iii) possess good flow properties and high binding capacity. Poly A coupled to S-MDA resin by diazotization generally meets these criteria since high levels of poly A are bound (40-50 mg/gm of S-MDA resin) and are not released by extensive washing with denaturing solvents such as 7M urea or 98% formamide. In addition, columns packed with resin have good flow rates and can be reused for numerous assays to assure quality control.

Poly U sequences have been isolated from RNase T₁ digests of HeLa nuclear and cytoplasmic RNAs on this poly A resin. As will be reported elsewhere, significant amounts of this cytoplasmic poly U are found covalently bound to polysomal mRNA¹². The poly U fractions isolated from digests of poly A containing RNA (but not from RNA lacking poly A) are contaminated with adenylyate-rich sequences when carrier poly U is added. Since this carrier addition is necessary for the maximal recovery of natural poly U, an additional separation step is necessary to remove this poly A. Because in the case of HeLa cells, at least, this poly A is much longer than poly U, this is easily achieved by gel electrophoresis.

The isolation of poly U containing RNA molecules will be an important extension of the methods described here. Some modifications of the present techniques will apparently be required since even in the absence of poly A containing RNA considerably less than a quantitative recovery of poly U containing RNA molecules have been attained thus far.

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