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Characterization of Poly(riboadenylic acid) Segments in L-Cell Messenger Ribonucleic Acid†

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ABSTRACT: The polyadenylate segments in L-cell mRNA have been characterized by the use of two-dimensional "fingerprinting" techniques. Digestion of poly(A)-containing mRNA with pancreatic ribonuclease and T_1 ribonuclease in high salt buffer releases the intact poly(A) segments; these are readily separable from the (A-)nCp, (A-)nGp, and (A-)nUp components which arise by digestion from the remainder of the molecule. Digestion of the same mRNA preparations with both enzymes, but in low salt buffer, results in the hydrolysis of the poly(A) segments to oligonucleotides with chain lengths $2 \geqslant 15$; these oligo(adenylic acid) components are also largely separable from the (A-)nCp, (A-)nGp, and (A-)nUp components. Fractionation of the alkali hydrolysis products of the oligoadenylates or of poly(A) released by digestion in high salt buffer did not reveal the presence of guanylic, cytidylic, or uridylic acid. This suggests that poly(A) segments in L-cell mRNA are homopolymers of adenylic acid. Using the same methods, no poly(A) could be detected in L-cell ribosomal RNA.

Poly(riboadenylic acid) [poly(A)] segments of up to 250 nucleotides in length have been reported to be covalently linked to heterogeneous nuclear RNA, eukaryotic messenger RNA (Lim and Canellakis, 1970; Darnell *et al.*, 1971b; Lee *et al.*, 1971; Edmonds *et al.*, 1971), and viral RNA (Kates, 1970; Lai and Duesberg, 1972).

The functional role of untranslated regions in eukaryotic mRNA such as the poly(A) tracts is uncertain at this time. However, there is evidence to suggest that poly(A) may be necessary for processing and transport of mRNA from the nucleus to the cytoplasm (Darnell *et al.*, 1971a). The recent demonstration that poly(A) tracts located at the 3′ terminus are longer in newly synthesized mRNA (Greenberg and Perry 1972a; Sheiness and Darnell, 1973) indicates that this region of the RNA may also serve to protect the informational part of the molecule from nucleolytic destruction during its life span in the cell. Further, the interaction of a heterogeneous population of mRNA molecules with ribosomes during translation or possibly with proteins during transport from the nucleus to the cytoplasm (Henshaw, 1968; Samarina *et al.*, 1968; Perry and Kelley, 1968) would require invariable regions in mRNA. Regions of the RNA which could be involved in RNA–protein interactions are likely to be in the untranslated regions at the chain termini, such as the poly(A) segments. Evidence for the involvement of mRNA poly(A) segments in mRNA–protein interactions has been described (Kwan and Brawerman, 1972; Blobel, 1973).

In order to gain some insight into the structure and post-transcriptional mechanism of the addition of poly(A) to mRNA, the isolation and nucleotide sequence analysis of the poly(A) segments in L-cell mRNA have been studied. The present report suggests that poly(A) tracts in chain lengths up to about 250 residues consist of only adenylic acid residues. No regions of the mRNA adjacent to the poly(A) segment are released with the poly(A) by nuclease digestion in high salt buffer.

Materials and Methods

Cells. Mouse L-cells were grown in Eagles minimum essential medium (Joklik-modified, Grand Island Biological Co.), containing 5% fetal calf serum.

Labeling of Cells. L-Cells ($1–2 \times 10^8$) in 30-ml cultures were grown for 3 to 4 hr at 37° in the presence of [2,8-^{3}H]-adenosine (150 μCi/ml) or carrier-free $^{32}PO_4$ (175 μCi/ml). Labeling of cells in the presence of $^{32}PO_4$ was done in phosphate-free medium.

In some experiments labeling of cells was carried out after an initial 30-min incubation in the presence of actinomycin D (0.05 μg/ml, Calbiochem) or in the presence of actinomycin D (0.05 μg/ml) and ethidium bromide (1 μg/ml, Calbiochem).

Cell Fractionation and Isolation of mRNA Containing Poly(A) Segments. Breakage of cells and preparation of post-mitochondrial supernatants were accomplished by procedures described previously (Penman *et al.*, 1963; Vesco and Penman,

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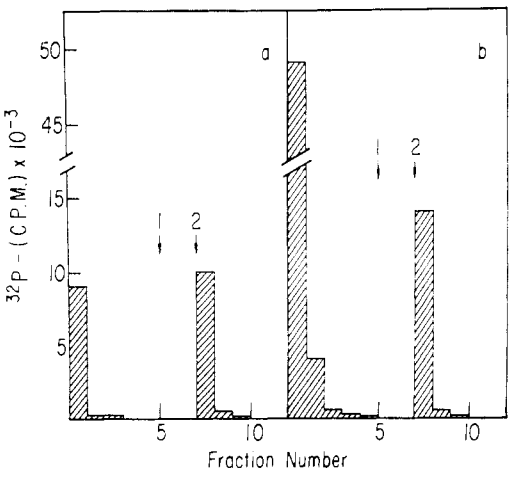

FIGURE 1: Affinity chromatography on Sepharose–poly(U) columns of polysomal RNA isolated from cultures grown in the presence (a) and absence (b) of actinomycin D. The arrows indicate the point of addition of buffer (0.1 M NaCl–0.01 M Tris-HCl (pH 7.4) (1) and water (2).

1969). Polysomes were isolated by centrifuging the post-mitochondrial supernatants for 2.5 hr through 40% sucrose in RSB[1] at 40,000 rpm in a SW 50 rotor. Alternatively, polysomes were isolated from the interphase between a 60% sucrose cushion (in RSB) and a 5–20% sucrose gradient (Lindberg and Persson, 1972).

RNA was isolated from polysomes by the method of Lee *et al.* (1971). The aqueous phase from the phenol deproteinization step was precipitated by the addition of three volumes of 95% ethanol and washed twice by resuspension and centrifugation in cold 95% ethanol (Nichols *et al.*, 1972). The RNA samples were placed briefly under vacuum to remove ethanol and stored until use at −20°.

RNA containing poly(A) was isolated from the polysomal RNA preparations by affinity chromatography on Sepharose 4B–poly(uridylic acid) columns. The preparation of Sepharose–poly(U) has been described (Lindberg *et al.*, 1972). RNA was dissolved in 0.5 ml of NETS[1] buffer and applied to a column of Sepharose–poly(U) which had been washed with 50 ml of NETS buffer. The microcolumn contained 2 ml of a Sepharose–poly(U) slurry in a glass wool-stoppered pasteur pipet. Unbound RNA was washed through the column with 10 ml of NETS buffer. Fractions of 2 ml were collected throughout. The column was then washed with 4 ml of buffer (0.1 M NaCl–0.01 M Tris-HCl (pH 7.4)) in order to remove sodium dodecyl sulfate and EDTA which were found to interfere with subsequent enzymic digestions and fractionations if they were present in samples containing small amounts of RNA. The RNA which remained bound to the column was then eluted with distilled water. No further RNA could be removed from the column by the use of a formamide buffer (Lindberg *et al.*, 1972). The radioactivity in each fraction was determined by counting a 10-μl aliquot in scintillation fluid (Nichols *et al.*, 1973). RNA was recovered by ethanol precipitation and washed twice with cold 95% ethanol.

Detection, Isolation, and Purification of Poly(A) Sequences. ^{32}P-Labeled poly(A) segments were isolated by digestion with both T_1 ribonuclease (0.2 unit; Sankyo Co.) and pancreatic ribonuclease (0.2 μg; Worthington Biochemicals) in 100 μl of high salt buffer (0.2 M NaCl–0.01 M Tris-HCl (pH 7.4)–0.01 M EDTA) for 1 hr at 37°. The digest was then made 1% in sodium dodecyl sulfate and 50 μg of carrier *Escherichia coli* tRNA was added. The RNA was precipitated with ethanol and subjected to electrophoresis in 5% polyacrylamide gels (Nichols *et al.*, 1973). The gels were cut into 1-mm slices and the poly(A) peak was detected by Čerenkov radiation using a Beckman scintillation counter. The poly(A) material was extracted from the gel by methods described previously (Nichols, 1970).

The presence of poly(A) segments in RNA was also determined by digestion of the RNA in 10 μl of high salt buffer containing 0.02 unit of T_1 ribonuclease and 0.02 μg of pancreatic ribonuclease, for 15 min at 37°. In this procedure the digestion products were fractionated by electrophoresis and homochromatography (Brownlee and Sanger, 1969). The thin layer plates used in the homochromatographic step were made with a mixture of DEAE-cellulose and cellulose (Machery, Nagel & Co.) in the proportions 1:7.5. The "homomix" consisted of a 3% partially hydrolyzed solution of RNA in 7 M urea. Material was eluted from the thin layer plates according to the procedure of Brownlee and Sanger (1969).

Digestion of ^{32}P-labeled RNA with both T_1 ribonuclease and pancreatic ribonuclease in low salt buffer (0.01 M Tris-HCl (pH 7.4)–0.001 M EDTA) was performed according to published procedures (Sanger *et al.*, 1965; Brownlee *et al.*, 1968). The digestion products were fractionated by electrophoresis and homochromatography as described above.

Sucrose Density Gradient Centrifugation. RNA samples in 1 ml of buffer (0.01 M EDTA–0.01 M NaCl–0.01 M Tris-HCl (pH 7.5)) were layered over linear 30–60% sucrose gradients (in the same buffer) and centrifuged in an SW 25 rotor at 6° for 16 hr. Fractions were collected from the bottom of the tube and assayed for Cl_3CCOOH-precipitable counts.

Isolation of L-cell Virus Low Molecular Weight RNA. The 4S and 7S RNA components of purified L-cell virus were isolated as described previously (Nichols *et al.*, 1973). ^3H-Labeled 4S and 7S components were detected in polyacrylamide gels following electrophoresis by treating each 1-mm slice with Protosol (New England Nuclear) prior to counting in a toluene-based scintillation fluid.

Alkali Hydrolysis of RNA and Poly(A). RNA samples were hydrolyzed in 0.2 N NaOH for 18 hr at 37°. The resulting nucleoside monophosphates were resolved by paper electrophoresis at pH 3.5 (Sanger *et al.*, 1965).

Results

Fractionation of L-cell polysomal RNA on Sepharose–poly(U) columns results in the separation of those ribonucleates containing covalently bound poly(A) regions from those which do not contain extensive poly(A) regions. Figure 1 illustrates the separation of these two RNA classes. The material which is not bound to the column is largely ribosomal RNA since incubation of the cells in low levels of actinomycin D (Figure 1a) prior to the addition of $^{32}PO_4$ results in a much decreased incorporation of radioactivity into this fraction, whereas the RNA bound to the column shows a much smaller decrease in the level of incorporated radioactivity. Actinomycin D in the concentrations employed in this experiment (0.05 μg/ml) has been shown to preferentially suppress ribosomal RNA synthesis (Perry, 1963). When

[1] Abbreviations used are: (A-)nCp, (A-)nGp, and (A-)nUp, oligonucleotides containing from 1 to 5 adenylic acid residues with a 3′-terminal cytidylic acid, guanylic acid, and uridylic acid residue, respectively; RSB, 0.01 M NaCl–0.01 M Tris-HCl (pH 7.2)–0.0015 M $MgCl_2$; NETS, 0.1 M NaCl–0.01 M EDTA–0.01 M Tris-HCl (pH 7.4)–0.2% sodium dodecyl sulfate.

TABLE I: Composition of Sepharose–Poly(U) Column Fractions.[a]

Nucleotide[b]	%
I. Unbound	
Up	25.1
Cp	23.1
Gp	34.0
Ap	17.8
II. Water Eluent	
Up	29.2
Cp	18.9
Gp	24.4
Ap	27.5

[a] The nucleoside 2′ (3′) monophosphates were resolved by paper electrophoresis at pH 3.5 (see Materials and Methods). The position of the nucleotides was determined by autoradiography and each nucleotide area was excised and counted in a toluene-based scintillation fluid (Nichols *et al.*, 1973). [b] The values represent the mean of two independent determinations and are not corrected for C → U deamination (5%).

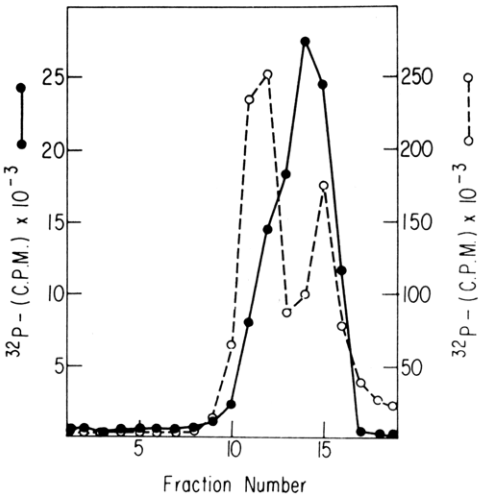

FIGURE 2: Sucrose density gradient profile of RNA fractionated by Sepharose–poly(U) chromatography. Fractions 1 and 2 (unbound RNA) and 8 and 9 (bound RNA) from the experiment illustrated in Figure 1b were pooled separately and the ethanol-precipitated RNA samples were redissolved and run in parallel gradients (see Materials and Methods). Cl_3CCOOH-precipitable counts of the bound (●) and unbound (○) RNA preparations in the gradient are plotted together.

cells were preincubated with both ethidium bromide and actinomycin D, the results were identical with those where just actinomycin D was employed (as in Figure 1a). This eliminates the possibility of mitochondrial RNA contamination in the preparations (Zylber *et al.*, 1969).

Sucrose density gradient fractionation of the two classes of RNA derived from cultures labeled in the absence of actinomycin D and separated by Sepharose–poly(U) chromatography (illustrated in Figure 1b) is shown in Figure 2. Messenger RNA which binds to the column is quite heterogeneous with a peak intermediate between the 28S and 18S ribosomal RNA components of the material which does not bind to the column.

The nucleotide compositions of the Sepharose–poly(U) bound and unbound RNAs are quite different; the mRNA (bound) contains a higher proportion of adenylic acid compared to the unbound fraction (Table I). The values for the nucleotide composition of rRNA (unbound) are similar to those previously reported by other workers (Lane and Tamaoki, 1967; Faras and Erikson, 1969).

The presence of poly(A) segments in mRNA eluted from Sepharose–poly(U) columns could be demonstrated by digestion of the RNA with T_1 and pancreatic ribonuclease in high salt buffer (see Materials and Methods), followed by fractionation of the digestion products using electrophoresis and homochromatography (Figure 3). The (A-)nGp, (A-)nCp, and (A-)nUp[1] components are well separated from the poly(A) which, because of its larger size, remains at the origin following the second-dimensional homochromatographic step. No large poly(A) segments could be detected if the same procedure was performed with unbound rRNA from Sepharose–poly(U) columns (data not shown). The poly(A) segments in the mRNA accounted for 8–10% of the total ^{32}P radioactivity when assayed by Cl_3CCOOH precipitation or Sepharose–poly(U) chromatography following digestion in high salt buffer.

It has previously been demonstrated that phosphodiester linkages involving adenylic acid residues become susceptible to the action of pancreatic ribonuclease under conditions of low ionic strength or suitably high concentrations of the enzyme (Lane and Butler, 1959; Beers, 1960). Thus, if digestion with both T_1 and pancreatic ribonuclease takes place in low salt buffer, the poly(A) segments in the RNA become susceptible to enzymic digestion resulting in oligoadenylates of varying sizes (Figure 4a). The oligoadenylates move in a unique position between the (A-)nCp and (A-)nGp components. Increasing the time of nuclease digestion results in the appearance of shorter oligoadenylates (data not shown) without detectably affecting the proportions of the (A-)nCp, (A-)nGp, and (A-)nUp components. Hydrolysis of the oligoadenylates with alkali showed that they contained only adenylic acid.

Figure 4b shows the separation of products resulting from digestion with T_1 and pancreatic ribonuclease in low salt buffer of Sepharose–poly(U) unbound rRNA. In this case, no oligoadenylates are evident, demonstrating that the

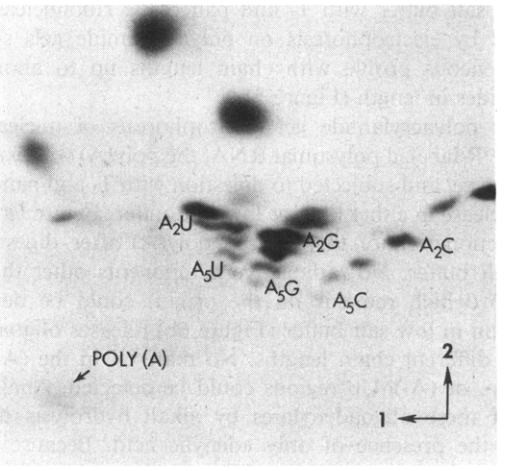

FIGURE 3: Autoradiograph of the electrophoretic–homochromatographic separation of a T_1 and pancreatic ribonuclease digest of mRNA performed in high salt buffer: (1) direction of electrophoresis in the first dimension; (2) direction of homochromatography in the second dimension.

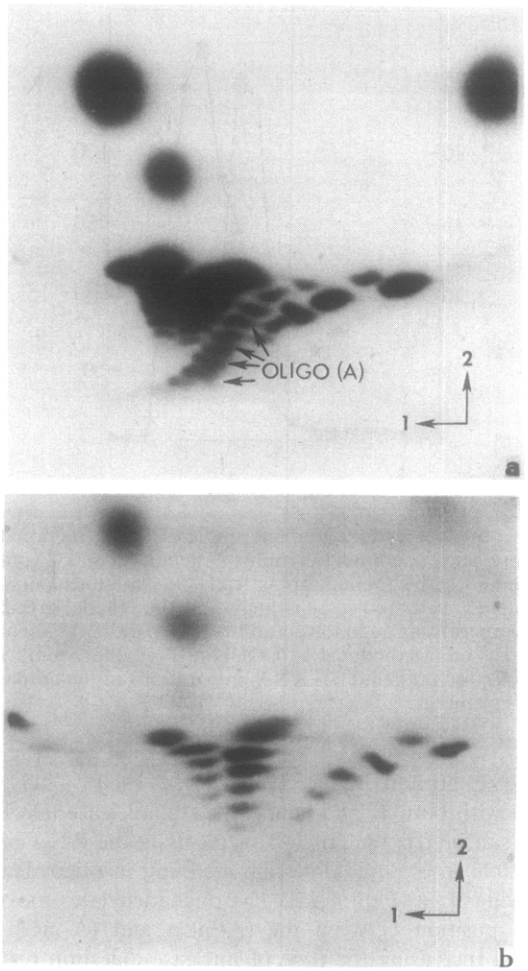

FIGURE 4: Autoradiographs of the electrophoretic–homochromatographic separations of T_1 and pancreatic ribonuclease digests of RNA performed in low salt buffer: (a) mRNA; (b) rRNA; (1) direction of electrophoresis in the first dimension; (2) direction of homochromatography in the second dimension

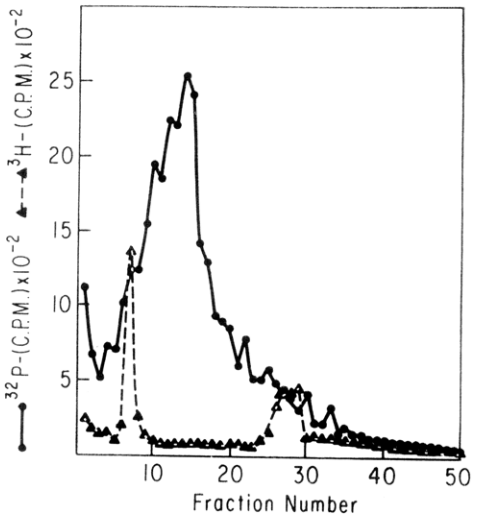

FIGURE 5: Polyacrylamide gel electrophoresis of polysomal RNA digested with T_1 and pancreatic ribonuclease in high salt buffer. Direction of electrophoresis is from left to right in relation to the figure: ^{32}P-labeled poly(A) (●) and tritiated 7S (chain length 280) and 4S (chain length 80) ribonucleates from L-cell virus (▲).

Sepharose–poly(U) column is selectively binding those species of RNA which contain large poly(A) segments.

Poly(A) segments liberated by digestion of polysomal RNA in high salt buffer with T_1 and pancreatic ribonuclease and purified by electrophoresis on polyacrylamide gels show a heterogeneous profile with chain lengths up to about 250 nucleotides in length (Figure 5).

After polyacrylamide gel electrophoresis of nuclease digested ^{32}P-labeled polysomal RNA, the poly(A) was extracted from the gel and subjected to digestion with T_1 and pancreatic ribonuclease in either high or low salt buffer. Figure 6a shows the fractionation of the isolated poly(A) after digestion in high salt buffer. No radioactive components other than the poly(A) (which remains on the origin) could be detected. Digestion in low salt buffer (Figure 6b) releases oligoadenylates of different chain lengths. No material in the (A-)nGp, (A-)nCp, or (A-)nUp regions could be detected. Analysis of each of these oligoadenylates by alkali hydrolysis demonstrated the presence of only adenylic acid. Because of the methods of analysis employed, it could not be determined which if any of those components had cyclic end groups. With oligoadenylates greater than 10 nucleotides in chain length, the recovery of radioactive material from the thin layer plate for subsequent analyses was decreased, and it was difficult, therefore, to rule out the possibility that these components might contain guanylic, cytidylic, or uridylic acid at their 3′ ends. However, when the time of digestion was increased, smaller oligonucleotides (<10 nucleotides chain length) were obtained and none of these moved in positions characteristic of the (A-)nGp, (A-)nCp, or (A-)nUp components. In addition, nucleotide composition studies on poly(A) purified by polyacrylamide gel electrophoresis showed that adenylic acid constituted at least 99.0% of the nucleotides present. Similarly high values for adenylic acid have been reported for HeLa cell mRNA poly(A) tracts (Edmonds *et al.*, 1971).

Discussion

Poly(A) segments in chain lengths up to about 250 nucleotides in length are released from L-cell mRNA, but not rRNA, by nuclease digestion in high salt buffer. Poly(A) released in this way and purified by polyacrylamide gel electrophoresis appears to contain only adenylic acid. No mono- or oligonucleotides other than oligoadenylates are released by nuclease digestion in low salt buffer of polyacrylamide gel electrophorectically purified poly(A). These results indicate that regions of the RNA preceding the poly(A) segments are not released with the poly(A) in the conditions employed. That region of the mRNA preceding the poly(A) segments is of interest because the nucleotide sequence may be important for recognition by the poly(A) synthetases (Edmonds and Abrams, 1960; Niessing and Sekeris, 1973). If this is the case, it is likely that all species of mRNA would have a common sequence at their 3′ ends following transcription. Digestion of mRNA under more partial conditions, or digestion of mRNA–protein complexes (Kwan and Brawerman, 1972; Blobel, 1973) followed by studies such as those described here, may make it possible to determine the nature of the supposedly untranslated regions preceding the poly(A) in cellular mRNA molecules.

Poly(A) in Rous sarcoma virus has been shown to consist of repeating units of between 10 and 40 adenylate residues followed by guanylic, cytidylic, or uridylic acid (Horst *et al.*, 1972). It will be of interest to determine if the presence of

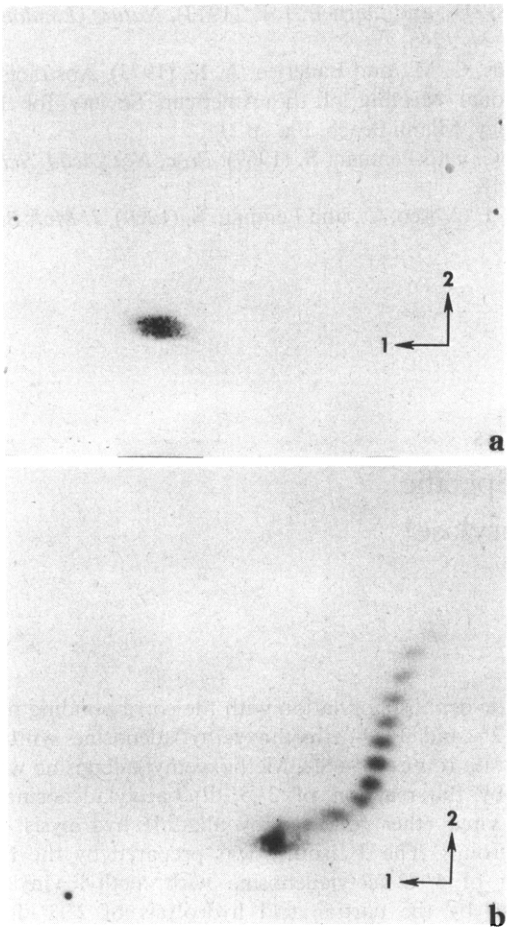

FIGURE 6: Autoradiographs of the electrophoretic–homochromatographic separations of T_1 and pancreatic ribonuclease digests of poly(A) isolated by polyacrylamide gel electrophoresis: (a) high salt buffer digest; (b) low salt buffer digest; (1) direction of electrophoresis in the first dimension; (2) direction of homochromatography in the second dimension.

nucleotides besides adenylic acid is a general feature of oncornavirus poly(A) segments, and, likewise, to determine if cellular mRNA molecules besides those of the L-cell have poly(A) segments which contain only adenylic acid. It is worth noting in this regard that nucleotides other than adenylic acid are thought to be present in poly(A) tracts of globin mRNA (Hunt, 1973), and this may indicate a difference in the poly(A) tracts among cellular mRNAs. However, previous studies on L-cell mRNA have shown that essentially all mRNA species have poly(A) segments covalently attached to the 3′ end of the molecules (Molloy *et al.*, 1972; Greenberg and Perry, 1972b) and no evidence for heterogeneity in these poly(A) tracts has been noted in this study.

Although most poly(A) has been shown to be present in covalent linkage at the 3′ ends of viral and mRNA molecules, naturally occurring poly(A) can also be present in another form. Whereas reovirus mRNA does not contain poly(A) (Stoltzfus and Banerjee, 1973), it has been shown that reovirions contain numerous short chain length oligoadenylates which are thought to be the result of reiterative "copying" of short sequences of uridylate residues in the genome RNA by an RNA polymerase (Nichols *et al.*, 1972). Thus, it would appear that the mechanism of synthesis of reovirus poly(A) is quite different from that of poly(A) covalently attached to viral and messenger RNA.

Acknowledgments

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Added in Proof

Following submission of this manuscript, a paper appeared by Molloy and Darnell (1973) which showed, using methods different from those employed in this study, that poly(A) tracts in Hela cell mRNA also consisted of only adenylic acid residues.

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2′-*O*-(α-Methoxyethyl)nucleoside 5′-Diphosphates as "Single-Addition" Substrates in the Synthesis of Specific Oligoribonucleotides with Polynucleotide Phosphorylase†

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ABSTRACT: In the presence of adenosine trinucleotide and 2′(3′)-*O*-(α-methoxyethyl)adenosine 5′-diphosphate, polynucleotide phosphorylase catalyzes the addition of only one nucleotide residue to the oligonucleotide to form adenosine tetranucleotide containing an α-methoxyethyl substituent at its terminus. The terminal nucleoside of this product has been removed by alkaline hydrolysis and shown to be 2′-*O*-(α-methoxyethyl)adenosine. In addition, the mixed 2′- and 3′-*O*-(α-methoxyethyl)adenosine 5′-diphosphates have been separated and characterized, and the conclusion that the 2′ isomer is the enzymatically active species has been confirmed. The structures of these two substituted nucleoside diphosphates were assigned by comparing the chromatographic and mass spectral properties of the products obtained from them by enzymatic dephosphorylation with the corresponding properties of 2′- and 3′-*O*-(α-methoxyethyl)adenosine synthesized by separate routes. 2′-*O*-(α-Methoxyethyl)adenosine was obtained by the reaction of 3′,5′-di-*O*-acetyladenosine with methyl vinyl ether followed by alkaline hydrolysis of the acetyl groups. The 3′ isomer was prepared by the limited reaction of 5′-*O*-acetyladenosine with methyl vinyl ether and also by the partial acid hydrolysis of 2′,3′-di-*O*-(α-methoxyethyl)adenosine. The mono(α-methoxyethyl) derivatives of each of the other three common ribonucleoside 5′-diphosphates can also be separated into two species and it is presumed that the enzymatically active species in each case is the corresponding 2′ isomer.

A new approach to the synthesis of polynucleotides of defined sequence has recently been developed in this laboratory (Mackey and Gilham, 1971). The basis of the method lies in the use of a nucleotide polymerizing enzyme with chemically modified substrates where the chemical modification is such that it permits the addition of only one nucleotide at a time to a growing polynucleotide chain. The particular case under study is that of polynucleotide phosphorylase with nucleoside 5′-diphosphates containing an *O*-(α-methoxyethyl) substituent group. This modification fulfills all the requirements for the realization of such a synthetic method: (i) the group is stable under the conditions of the enzyme reaction, (ii) it permits the addition of one nucleotide to an acceptor oligonucleotide and prevents the addition of a second, (iii) the group is readily removed under chemical conditions that do not affect the structural integrity of the oligonucleotide product, and (iv) after the removal of the blocking group the product is then available for a second single addition, and so on. This paper contains both a detailed description of the study reported in the above preliminary communication and a discussion of the evidence supporting the contention that, in the substrate, 2′(3′)-*O*-(α-methoxyethyl)nucleoside 5′-diphosphate, it is the 2′ isomer that is the enzymatically active species.

Reaction of α-Methoxyethyladenosine Diphosphates with Adenosine Trinucleotide. The limited reaction of adenosine 5′-diphosphate with methyl vinyl ether yields a mixture of the disubstituted and monosubstituted derivatives together with unreacted nucleotide and these can be separated readily by paper chromatography. The monosubstituted product, on further chromatography, separates into a faster moving and a slower moving component and these are shown below to be the 2′ isomer (II) and the 3′ isomer (I), respectively (Figure 1). These derivatives have been tested both separately and in combination in addition reactions using adenosine trinucleotide as the acceptor oligonucleotide. The 2′ isomer was found to permit the addition of a single nucleotide to the acceptor molecule to the same extent as a mixture of the 2′ and 3′ isomers, whereas the 3′ isomer alone gave essentially no addition. These reaction mixtures were analyzed on columns of a polystyrene anion exchanger using a solvent system containing 20% ethanol and a linear gradient of chloride ion (Ho and Gilham, 1973). In this system the starting materials

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