THE PRESENCE OF POLYADENYLATE SEQUENCES IN THE RIBONUCLEIC ACID OF A HIGHER PLANT

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SUMMARY:

Rice callus tissue in suspension culture was labeled in vivo with $^{32}PO_4^{3-}$. Approximately 4% of the total RNA extracted from the tissue could be bound to nitrocellulose filters in the presence of 0.5 M KCl. The base composition of this material was: A, 30.6; U, 32.7; G, 18.0; and C, 18.6%. If the RNA was digested with both T_1 and pancreatic ribonucleases prior to binding, the base composition of the bound material was: A, 97.8; U, 0.0; G, 1.6; and C, 0.5%. About 14% of the RNA isolated from purified polyribosomes could be bound to filters in 0.5 M KCl. It is concluded that higher plant RNA contains polyadenylate sequences.

INTRODUCTION:

Polyadenylate sequences covalently bound to mammalian mRNA (messenger RNA) have been known for some time (1). They have been found in vaccinia (2), in the RNA of tumor viruses (3), and in some single-stranded RNA viruses (4). Although their role in cellular metabolism is not clear, it has been suggested that they are concerned with the transport of mRNA from the nucleus to the cytoplasm (5) or that they control the life of the mRNA in the cytoplasm (6).

Although there have been no published reports of polyadenylate sequences from the RNA of higher plants, an adenylating enzyme has been reported in maize (7), and an RNA rich in adenosine has been reported in carrot and soybean tissue (8). This paper is concerned with the identification of polyadenylate from cultured rice tissue.

METHODS AND MATERIALS:

Rice Tissue - Callus tissue derived from rice root (Starbonnet) was

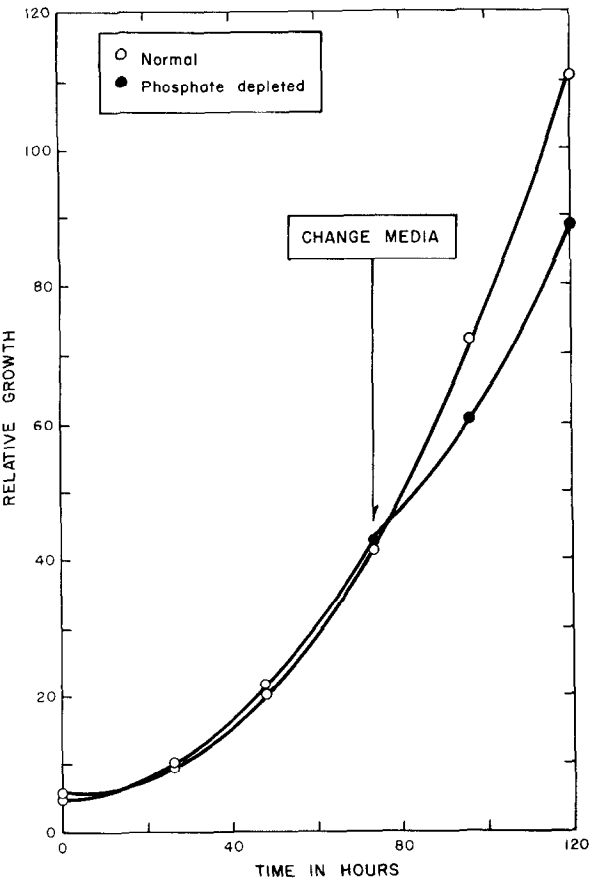

Figure 1. Effect of Altering Culture Medium on Subsequent Growth of Rice Tissue

All tissue was grown in normal medium for 72 hours. The control tissue was then transferred to fresh normal medium and the "phosphate depleted" tissue transferred to phosphate free medium. The maximum variation observed (duplicate samples) was 3.5%.

grown in liquid suspension medium as described by Lieb et al. (9). Growth measurements were made in Nephelo flasks. Tissue was partially depleted of phosphate 24 hours prior to labeling by incubation in a medium which contained 10.0 mM KCl, 7.0 mM $NaNO_3$, 1.0 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.37 µM $FeCl_3$, and 2% sucrose. Tissue was labeled with 0.5 - 1.0 mCi $^{32}PO_4^{3-}$ during "late log" phase and harvested by filtration on fiberglass filters.

Total RNA Extraction - Cells were homogenized with a Ten Broeck homogenizer in approximately 1 ml of buffer per gm fresh weight of cells. Ribonucleic acid was prepared from the homogenate by a modification of Edmonds and

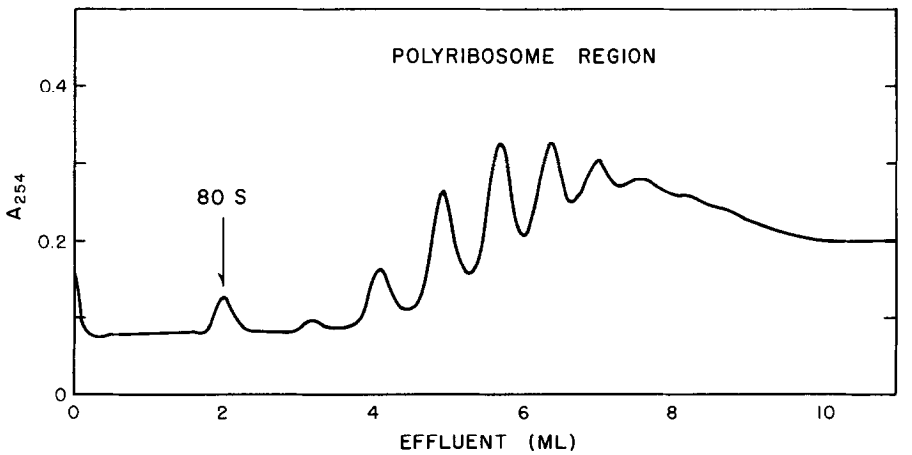

Figure 2. Sucrose Density Gradient Centrifugation of Polyribosomes Prepared from Tissue Incubated in Phosphate Free Medium

Rice tissue was incubated in the phosphate depletion medium for 72 hours. Polyribosomes were prepared, layered on a 15 - 30% sucrose gradient (containing 5 mM Mg^{2+} in resuspension buffer) and centrifuged for 1.5 hours at 40,000 RPM in a Spinco SW 41 at 2° C and the absorbance was monitored as previously described (13).

Caramela's (10) method II using 88% phenol and 25° C throughout. A buffer containing 100 mM Tris HCl pH 9.0, 10.0 mM KCl, and 0.5 mM $MgCl_2$ was used, and solid sodium dodecyl sulfate was added to a final concentration of 0.5%. After phenol extraction, the crude nucleic acid was purified by precipitating in 0.005 N HCl at 0° C, centrifuging for 10 minutes at 20,000 x g, discarding the supernatant fluid, and resuspending the precipitate in 5.0 mM NaCl adjusted to pH 10.6 with NaOH. This washing procedure was repeated three times to remove inorganic phosphate, presumably in the form of polyphosphate (11). After the final resuspension, the nucleic acid was adjusted to pH 8.0, and precipitated with 0.1 volume of 1% NaCl and 2.5 volumes of abs. ethanol at -20° C for 3 hours. The precipitate was centrifuged at 20,000 x g for 10 minutes, the supernatant fraction was discarded, and the nucleic acid was resuspended in distilled H_2O.

Polyribosomes and Polyribosomal RNA - Polyribosomes were prepared as previously described (12), except that the homogenization medium was buffered to pH 9.0. Sucrose density gradient centrifugation of the polyribosomes was per-

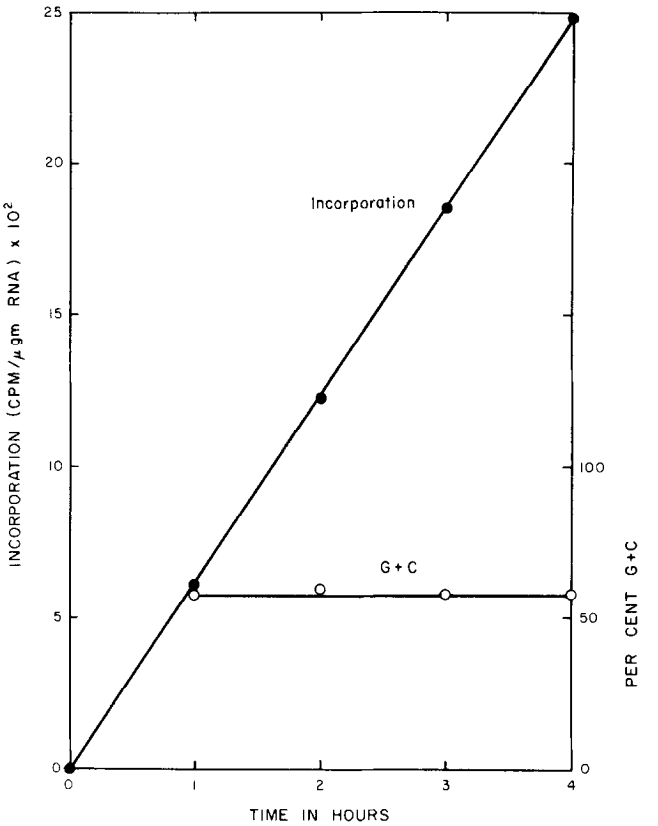

Figure 3. Effect of Time on Incorporation of $^{32}PO_4^{3-}$ into Total RNA and the Percentage of the Incorporation in G + C

Total RNA was purified as described under Methods. Radioactivity was determined by precipitating an aliquot at 0° with 10% Cl_3CCOOH, collecting the precipitate on a fiberglass filter, drying at 98° C, and counting in toluene basic scintillation fluid. Amount of RNA was determined by the orcinol color reaction (18). Another aliquot was hydrolyzed and the amount of incorporation into the various nucleotides determined as described under Methods. The quantity of RNA in a preparation was estimated by its absorption at 260 mμ.

formed as previously described (13). Polyribosomal RNA was extracted from the polyribosomes as described above, except that $CHCl_3$:phenol 1:1 (14) was used instead of 88% aqueous phenol.

Millipore Binding - Millipore binding was carried out as described by Brawerman et al. (15). Release of filter-bound RNA was carried out at 45° C in either 0.5% sodium dodecyl sulfate, 100 mM Tris HCl pH 9.0 (16) or 10.0 mM NaEDTA pH 9.0. Nucleic acid released from the filters was purified by either

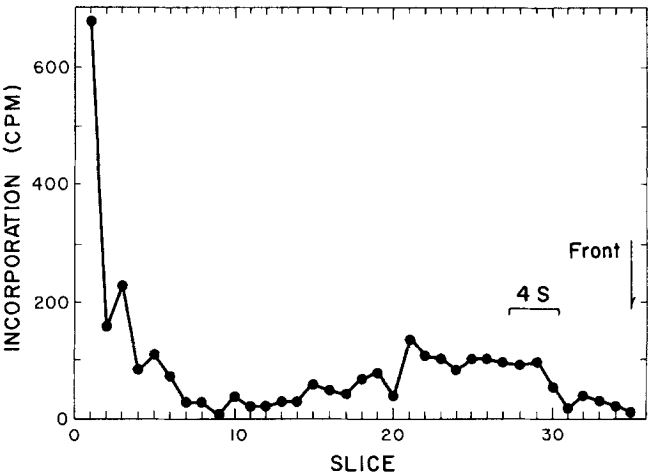

Figure 4. Electrophoretic Analysis of Poly A Obtained from a Total RNA Preparation

The RNA was digested with DNase, pancreatic and T_1 RNase, and bound to Millipore filters as described under Tables I and II. The nuclease resistant material was removed from the filters as described under Methods, and subjected to 10% polyacrylamide gel electrophoresis (5 mm dia.) at 5 ma/gel for 3-1/2 hours at 2° C (19). The front as marked by the bromphenol blue dye is noted. The approximate position of ^{14}C-tRNA (4S) on the gel after electrophoresis under identical conditions is also indicated. After electrophoresis, gels were removed from the glass tubing, sliced into 1 mm thick sections, and counted in "Protosol" as described in New England Nuclear Publication 5M-172M-480.

a combination of acid precipitation and ethanol precipitation or ethanol precipitation alone.

Base Composition - Ribonucleic acid was hydrolyzed with 0.3 N KOH for 18 hours at 37° C. The hydrolysate was neutralized with $HClO_4$, the salt removed by centrifugation at 0° C, and the bases assayed on Dowex-1 as described by Weinstein et al. (17). Incorporation into the various nucleotides was monitored by passing the column eluate through a flow cell in a Beckman LS-200 scintillation counter.

Miscellaneous - Deoxyribonuclease free of RNase activity was purchased from Worthington Biochemical Co. Pancreatic and T_1 RNases were purchased from Miles Research Products Division.

RESULTS:

Rice tissue was incubated in the phosphate free medium for 24 hours prior

TABLE I

Binding of Total and Polyribosomal Ribonucleic Acid to Nitrocellulose Filters

Type of RNA	% Filter bound
Total RNA prepared with 88% phenol	4.2
Total RNA prepared with 1:1 $CHCl_3$:phenol	4.4
Polyribosomal RNA prepared with 88% phenol	14.2
Polyribosomal RNA prepared with 1:1 $CHCl_3$:phenol	30.5

Fifty µl of RNA solution (4 - 6 µgm) was incubated with 20 µgm of ribonuclease-free deoxyribonuclease for 30 minutes at 37° C. One ml of 0.5 M KCl was added, and the tubes were allowed to stand for 5 minutes. The mixture was filtered through presoaked "Millipore" HAWP type filters, and the filters dried at 98° C for 30 minutes, and then counted in 10.0 ml of toluene-based scintillation fluid on a Beckman LS-100.

TABLE II

Base Composition of Total RNA after Various Treatments

Treatment	%A	%U	%G	%C
None	18.8	23.6	36.9	20.5
Filter bound	30.6	32.7	18.0	18.6
Pancreatic RNase, then filter bound	74.6	1.2	22.5	1.7
Pancreatic and T_1 RNase, then filter bound	97.8	0.0	1.6	0.5

Total RNase was treated with DNase as described under Table I. Pancreatic ribonuclease digestion was carried out at a ratio of 5 µgm ribonuclease to 1 µgm of RNA. Digestions were for 1 hour at 37° C. T_1 ribonuclease was used at a ratio of 10,000 units per mg RNA at 37° C.

to labeling with $^{32}PO_4^{3-}$ in order to insure a high specific activity. The phosphate free medium decreased the rate of growth (Figure 1). Polyribosomes were isolated from tissue that had been incubated in the phosphate free medium (Figure 2). It was found that the ultraviolet absorption pattern after sucrose density gradient centrifugation remained similar to that of control

TABLE III

Amount of Polyadenylate in RNA Bound to Filters

Type of RNA applied to filters	CPM bound
Total RNA	25,006
Total RNA after RNase digestion	5,720
Polyribosomal RNA	2,379
Polyribosomal RNA after RNase digestion	582

Both the total and polyribosomal RNA were treated with DNase before use. Filter binding, nuclease digestions, and counting were as described under Tables I and II. Digestion was with both pancreatic and T_1 ribonucleases.

tissue for at least 72 hours. Extension of the labeling periods from 1 - 4 hours made little difference in the quality of the RNA as determined by per cent G + C. However, the specific activity of the RNA increased in a linear fashion during this time (Figure 3). The above data suggested that the tissue was actively growing in the phosphate free medium during the period of interest.

The percentage of total RNA that was DNase resistant and could be bound to filters was approximately 4%. The percentage of the polyribosomal RNA which could be bound was much higher, and could be enhanced still more by employing the $CHCl_3$-phenol extraction procedure (Table I). The fraction of the total RNA which was filter bound had a lower percentage of G + C than did the total RNA. After digestion with pancreatic RNase, the A content of the bound material increased to >4.6%, and after digestion with both pancreatic and T_1 RNases the A content of the bound material was 98% (Table II). The percentage of polyadenylate in the filter-bound RNA was approximately the same for both total and polyribosomal RNA (Table III). Polyadenylate from whole cells was assayed for size distribution by polyacrylamide gel electrophoresis (Figure 4). This showed a heterogeneous pattern with an accumulation in the 4 - 6S regions of the gel.

DISCUSSION:

The occurrence of polyadenylate sequences in the RNA of higher plants has

been demonstrated. It appears that the RNA prepared from rice polyribosomes has a higher percentage of poly A containing segments than total RNA. Poly A in mammalian cells has been demonstrated to be attached to mRNA, and a similar situation now appears likely in higher plants.

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