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# Isolation and Characterization of Polyadenylate-Containing RNA from *Bacillus brevis*†

Nilima Sarkar,* Donna Langley, and Henry Paulus

ABSTRACT: A substantial fraction (30–40%) of pulse-labeled RNA from exponentially growing cells of *Bacillus brevis* contains polyadenylate sequences, as measured by adsorption to oligo(dT)-cellulose. The weight-average length of poly(A) tracts obtained after digestion with pancreatic and $T_1$ ribonucleases is 60 nucleotide residues. Susceptibility to degradation by snake venom phosphodiesterase after ribonuclease degradation indicates that the poly(A) sequences are located near the 3′ ends of the RNA chains, but that in 40% of the material at least one internal pyrimidine nucleotide residue intervenes between the poly(A) tract and the 3′-hydroxyl terminus. These pyrimidine nucleotides consist of 65% cytidylate and 35% uridylate residues. In the remaining RNA chains, the poly(A) sequence is directly at the 3′-terminus, but the possibility cannot be excluded that a small fraction of this material may contain a 3′-hydroxyl terminal guanylate residue. The weight-average sedimentation coefficient of poly(A)-containing RNA is 12.5 S, corresponding to a polynucleotide chain length of 800–900 residues. This is in a size range expected for messenger RNA, a possibility which is also supported by the observation that pulse-labeled RNA has a considerably higher poly(A) content than long-term labeled RNA.

Since the discovery of poly(A) sequences in mRNA from eukaryotic cells (Darnell et al., 1971; Edmonds et al., 1971; Lee et al., 1971), much thought has been given to their possible function, but no clear answer has emerged. One possible approach to this question is to study the distribution of poly(A) sequences in nature, especially in simpler systems. In this connection, the presence of such RNA species was recently reported in several prokaryotic systems. Very low levels of poly(A) RNA have first been described in *Escherichia coli* (Nakazato et al., 1975), but somewhat higher levels (up to 15%) have been found under different growth conditions (Srinivasan et al., 1975). Relatively short poly(A) sequences (about 15 nucleotides) have also been found in *Caulobacter crescentus* mRNA (Ohta et al., 1975, 1978). In this paper, we report the occurrence of relatively high levels (30–40%) of poly(A)-containing molecules among the rapidly labeled RNA species of *Bacillus brevis* and describe their characterization.

## Experimental Procedure

*Materials.* These were obtained from the following sources: [5-$^3$H]uridine (25 Ci/mmol) and [2,8-$^3$H]adenosine (16.8 Ci/mmol) were from New England Nuclear; pancreatic ribonuclease A (Grade R, 2900 units/mg) and bacterial alkaline phosphatase (Grade BAPF, 55 units/mg) were from Worthington; $T_1$ ribonuclease (6500 units/mg) was from Calbiochem; snake venom phosphodiesterase (1.5 units/mg) was from Boehringer; proteinase K was from Beckman; poly(adenylic acid) was from P-L Biochemicals; poly(cytidylic acid) was from Miles; tRNA (*Escherichia coli*) was from Schwarz-Mann; oligo(dT)-cellulose (Type T2) was from Collaborative Research; PEI-cellulose[1] sheets were from Brinkmann; cellulose thin-layer sheets (with fluorescent indicator) were from Eastman; heparin was from Organon; and sucrose (density gradient grade) was from Schwarz-Mann. rRNA was isolated by phenol extraction from ribosomes of *B. brevis,* either unlabeled or labeled with [$^3$H]uridine. $(Ap)_{\overline{70}}$Up, containing [$^{14}$C]adenosine (0.1 $\mu$Ci/$\mu$mol) and [$^3$H]uridine (10 $\mu$Ci/$\mu$mol), was prepared as described previously (Sarkar and Paulus, 1975).

*Bacterial Growth and Labeling Procedures. Bacillus brevis* ATCC 8185 was grown in the medium of Hanson et al. (1964) at 37 °C on a rotary shaker. Growth was monitored with a Klett-Summerson photoelectric colorimeter using the No. 42 filter. During early exponential growth (100–110 Klett units), culture samples (1 mL) were treated with [5-$^3$H]uridine or [2,8-$^3$H]adenosine (generally 1–10 $\mu$Ci). After 30 s, the samples were quickly chilled to 4 °C and treated with 25 mM $NaN_3$. The cells were collected by centrifugation at 10 000*g* for 5 min and washed once with growth medium containing 25 mM $NaN_3$.

*Isolation of Poly(A)-Containing RNA.* The washed pulse-labeled cells were suspended in 0.38 mL of a solution containing 80 mM Tris-HCl, pH 9.0; 10 mM EDTA; 20 mM 1,10-phenanthroline; 0.5% sodium dodecyl sulfate; 0.2 mg/mL heparin; and 0.23 mg/mL proteinase K. The mixture was in-

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[1] Abbreviations used: PEI-cellulose, polyethylenimine-impregnated cellulose; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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cubated at 37 °C for 20 min, then supplemented with 4 M NaCl to a final concentration of 0.5 M, and added to 10 mg of oligo(dT)-cellulose that had been washed with 1 mL of 1 N NaOH and twice with 5 mL of 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5, in a sterile conical centrifuge tube. After heating at 60 °C for 4 min (a step which was occasionally omitted without effect on the results), the suspension was shaken at room temperature for 30 min, and the cellulose was collected by centrifugation, washed four times by suspension in 4 mL of 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5, and then eluted with two 1-mL portions of water by incubating at 45 °C for 15 min.

*Preparation of Poly(A) Tracts by RNase Digestion.* Fragments of pulse-labeled RNA containing the poly(A) moiety were isolated by RNase digestion of material adsorbed to oligo(dT)-cellulose under conditions of high ionic strength to prevent cleavage between adenylate residues (Beers, 1960). Pulse-labeled RNA, adsorbed to oligo(dT)-cellulose and washed with high salt buffer as described above, was suspended in 1 mL of 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 2 mM EDTA, and incubated at 25 °C for 30 min with 40 μg of pancreatic RNase, 0.31 μg of $T_1$ RNase, or both. The cellulose was collected by centrifugation, washed twice with 4-mL portions of 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5, and then eluted at 45 °C with two 1-mL portions of water containing 34 μg of poly(A) as carrier. The eluted samples were then evaporated to dryness in a vacuum for further processing.

*Polyacrylamide Gel Electrophoresis.* Electrophoresis in 14% polyacrylamide gels was carried out essentially by the procedure of Peacock and Dingman (1968). The isolated poly(A) tracts were redissolved in 0.04 mL of buffer containing 40 mM Tris–acetate, pH 7.2, 20 mM sodium acetate, 1 mM EDTA, and 0.4% sodium dodecyl sulfate. The sample was clarified by centrifugation, and a portion (0.03 mL) was mixed with a small amount of Bromphenol blue and layered on 6 × 90 mm cylindrical polyacrylamide gels. Electrophoresis was carried out in the same buffer at 60 V (5 mA/gel) until the marker dye had reached the bottom of the tubes (about 3 h). The gels were then sliced into 2-mm segments with the device of Matsumara and Noda (1973), and the slices were incubated at 37 °C for 18 h in 0.2 mL of 0.5 N NaOH. After neutralization with acetic acid, radioactivity was determined in a liquid scintillation spectrometer using the scintillation fluid of Anderson and McClure (1973).

The electrophoretic mobility of the poly(A) tracts was compared with that of the synthetic polymer $(Ap)_nUp$, labeled with [$^{14}$C]adenosine and [$^3$H]uridine, which was run in a parallel gel. From the ratio of tritium to carbon-14 in each gel fraction and the known specific activities of the two isotopes in the polymer, it was possible to calculate the relative number of adenylate and uridylate residues in each fraction, which corresponds to the polynucleotide chain length.

*Sucrose Gradient Centrifugation.* A proteinase K lysate (0.24 mL) of *B. brevis* which had been pulse-labeled with [$^3$H]uridine was supplemented with 160 μg of *E. coli* tRNA and precipitated with 75% ethanol. The precipitate was redissolved in 0.1 mL of 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5, precipitated a second time, and then dissolved in a sterile buffer (0.1 mL) which contained: 10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 0.1 M NaCl; 0.05% sodium dodecyl sulfate; and 0.2 mg/mL rRNA. This sample was layered onto 5 mL of a linear gradient (5–20%) of sucrose in 10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 0.1 M NaCl; 0.05% sodium dodecyl sulfate; and 20 μg/mL heparin. After centrifugation for 16 h at 16 500 rpm in a Beckman SW50.1 rotor at 24 °C, the bottoms of the tubes were punctured and 0.1-mL fractions were collected. Alternate fractions were analyzed for total radioactivity and for radioactivity adsorbed to oligo(dT)-cellulose in 0.5 M NaCl and eluted with water at 45 °C.

*Alkaline Hydrolysis and Analysis of Products.* [$^3$H]Uridine-labeled RNA samples were evaporated to dryness in conical centrifuge tubes and redissolved in 0.03 mL of 0.5 N NaOH. After 18 h at 37 °C, the hydrolysates were diluted to 0.5 mL with water, supplemented with 0.1 μmol each of 3′-CMP, 3′-UMP, cytidine, and uridine, and neutralized with 1 N acetic acid. After evaporation to dryness in a vacuum, the samples were dissolved in 0.02 mL of water and applied as a thin streak near one edge of a PEI-cellulose thin-layer plate. Development of the chromatogram was for a distance of 12 cm in either 1 M acetic acid ($R_f$ values: 3′-UMP, 0.12; 3′-CMP, 0.54; cytidine and uridine, 0.95) or 1 M HCOOH ($R_f$ values: 3′-UMP, 0.07; 3′-CMP, 0.83). The cellulose was scraped off the zones corresponding to the nucleotide standards and suspended in 1 mL of 1 M LiCl, and radioactivity was determined in the scintillation fluid of Anderson and McClure (1973).

*Estimation of 3′-Terminal Adenosine Residues.* Cells of *B. brevis* were pulse-labeled with a high level of [$^3$H]adenosine (50 μCi/mL) and the poly(A) tracts were isolated after treatment with pancreatic RNase (0.4 mg/mL) as described in earlier sections. The poly(A) tracts were hydrolyzed in 0.03 mL of 0.3 N NaOH at 37 °C for 18 h, diluted with water, and neutralized to pH 9 with $H_3BO_3$, evaporated to dryness, and redissolved in 0.04 mL of water containing 0.1 μmol each of adenine and adenosine. The sample was applied as a thin streak near one edge of a cellulose thin-layer sheet that had been wetted with 0.1 M sodium borate buffer, pH 9.2, and subjected to electrophoresis in this buffer at 700 V for 72 min. During this time, 3′-AMP, adenosine, adenine, and Bromphenol blue had migrated 13.5, 5.5, 0, and 13 cm, respectively. The thin-layer sheet was divided into 1-cm zones, and the cellulose from each zone was scraped into a scintillation vial containing 1 mL of water and its radioactivity determined in the scintillation fluid of Anderson and McClure (1973).

## Results

*Isolation of Poly(A)-Containing RNA.* When pulse-labeled RNA from *B. brevis* was fractionated by adsorption to oligo(dT)-cellulose, about 30% of the radioactivity was found to be bound at 0.5 M NaCl and eluted at low ionic strength (Figure 1). Adsorption to oligo(dT)-cellulose appeared to be a specific process, since only a small fraction of the originally unadsorbed material was bound when treated a second time with the cellulose derivative, whereas most of the material eluted at low ionic strength was readsorbed during a second cellulose treatment (Table I). Prior treatment of oligo(dT)-cellulose with poly(A) reduced the amount of pulse-labeled RNA bound by 70%, but treatment with poly(C) had no effect, suggesting that the observed binding was due to the presence of poly(A) sequences in pulse-labeled RNA. RNA pulse-labeled with [$^3$H]uridine was bound to nearly the same extent as [$^3$H]adenosine-labeled RNA,[2] but ribosomal RNA was not retained by oligo(dT)-cellulose (Table I).

In order to confirm that the binding of pulse-labeled RNA to oligo(dT)-cellulose was due to the presence of poly(A) sequences, the adsorbed material, after elution at low ionic strength, was treated with pancreatic RNase. As shown in

[2] Some variability was observed in the extent of binding of [$^3$H]uridine-labeled RNA to oligo(dT)-cellulose, perhaps on account of the greater vulnerability of pyrimidine-containing sequences to nuclease digestion during RNA isolation.

TABLE I: Specifity of Binding to Oligo(dT)-cellulose.[a]

| RNA sample | radioact. eluted at high salt (cpm) | radioact. eluted at low salt | |
|---|---|---|---|
| | | cpm | % |
| (A) RNA pulse labeled with [$^3$H]Ado | 74 200 | 36 200 | 36 |
| (B) high-salt eluate from A | 31 600 | 2 240 | 7 |
| (C) low-salt eluate from A | 3 380 | 18 300 | 84 |
| (D) as A, but oligo(dT)-cellulose treated with poly(A)[b] | 95 400 | 11 200 | 11 |
| (E) as A, but oligo(dT)-cellulose treated with poly(C)[c] | 76 000 | 41 400 | 35 |
| (F) RNA pulse labeled with [$^3$H]Urd | 282 500 | 120 700 | 30 |
| (G) rRNA labeled with [$^3$H]Urd | 27 000 | 20 | 0 |

[a] RNA was adsorbed to oligo(dT)-cellulose (10 mg) in 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, and the cellulose was washed with the same buffer and then with water as described under Experimental Procedure. [b] Before use, oligo(dT)-cellulose was treated with 0.46 mg of poly(A) in 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and washed with the same buffer. [c] Before use, oligo(dT)-cellulose was treated with 0.72 mg of poly(C) in 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and washed with the same buffer.

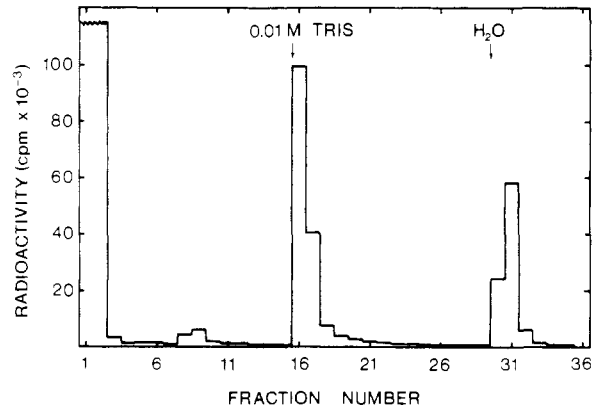


FIGURE 1: Chromatography of pulse-labeled RNA on oligo(dT)-cellulose. A proteinase K lysate (0.4 mL; 1.34 × $10^6$ cpm) of [$^3$H]adenosine-labeled cells (see Experimental Procedure) was applied to a column containing 20 mg of oligo(dT)-cellulose equilibrated with 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5. The column was first washed with 16 mL of this buffer, and then with 14 mL of 10 mM Tris-HCl, pH 7.5, and finally with 16 mL of water. Fractions of about 1 mL were collected. The *arrows* indicate the positions of buffer changes.

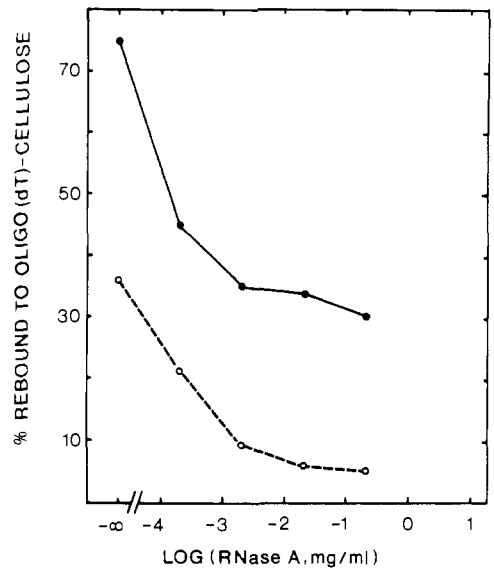


FIGURE 2: Digestion of poly(A)-containing RNA with pancreatic RNase. Poly(A)-RNA was isolated from cells pulse labeled either with [$^3$H]-adenosine or [$^3$H]uridine as described under Experimental Procedure. Samples of poly(A)-RNA (7200 or 2300 cpm, respectively, of [$^3$H]-adenosine- or [$^3$H]uridine-labeled RNA) were incubated at 25 °C for 15 min at the indicated concentrations of pancreatic RNase in 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, and 2 mM EDTA. Oligo(dT)-cellulose (10 mg) was then added to each sample and the fraction of radioactivity bound at 0.5 M NaCl was determined: (●—●) RNA labeled with [$^3$H]adenosine; (○- - -○) RNA labeled with [$^3$H]uridine.

Figure 2, RNA labeled with [$^3$H]uridine was almost completely converted to a form which was no longer retained by oligo(dT)-cellulose. In contrast, about 30% of [$^3$H]adenosine-labeled RNA retained its ability to bind to oligo(dT)-cellulose even after exhaustive RNase digestion, indicating that a substantial fraction of the adenylate residues were present as a repeating sequence. The fact that a 100-fold increase in RNase concentration (from 2 to 200 μg/mL) reduced the fraction of labeled RNA bound to oligo(dT)-cellulose by only 15% showed that RNase digestion was essentially complete. At 2 μg/mL RNase, no difference was seen in the amount of [$^3$H]adenosine-labeled RNA remaining regardless of whether the digestion was carried out at 0.15 or 0.5 M salt (not shown).

*Length of the Poly(A) Sequences.* The size of the poly(A) sequences was estimated by polyacrylamide gel electrophoresis of material isolated on oligo(dT)-cellulose and exhaustively digested with a mixture of pancreatic and $T_1$ RNases. Figure 3 shows that the RNase-resistant fraction migrated as a somewhat skewed peak, with a considerable amount of higher molecular weight material trailing after the major component. The length of poly(A) in each gel fraction was estimated by comparison with the electrophoretic mobility of the synthetic polymer $(Ap)_nU_p(\bar{n} = 70$, range = 15–250), labeled with [$^{14}$C]adenosine and [$^3$H]uridine, whose molecular weight at any position in the gel could be calculated from the ratio of $^{14}$C to $^3$H. The weight-average length of the poly(A) tracts was found to be 60 nucleotides, while the number-average length was 48 nucleotides with 75% of the molecules at 50 ± 14 nucleotide residues. A similar length distribution was seen with poly(A) tracts isolated after treatment with $T_1$ RNase alone (not shown).

*Location of the Poly(A) Sequences in the RNA Chains.* The position of the poly(A) tracts in the RNA molecules was examined by assaying their susceptibility to exonucleolytic degradation by snake venom phosphodiesterase after treatment with $T_1$ or pancreatic RNase. The rationale for this procedure was based on the fact that snake venom phosphodiesterase can mediate exonucleolytic hydrolysis only of RNA chains with free 3′-hydroxyl termini. Since $T_1$ and pancreatic RNases yield oligonucleotides terminated with a 3′-phosphate at the site of cleavage, poly(A) sequences which have suffered an RNase split between the ultimate adenylate residue and the 3′ end of the RNA chain should be resistant to degradation by snake venom phosphodiesterase unless treated with alkaline phos-

TABLE II: Products of Alkaline Hydrolysis of Poly(A)-Containing Sequences Derived from RNA Pulse-labeled with [$^3$H]Uridine.

| RNA sample | treatment | distribution of radioact. in alkaline hydrolysate[a] 2′(3′)-UMP % of radioact. | 2′(3′)-UMP rel molar amt | 2′(3′)-CMP % of radioact. | 2′(3′)-CMP rel molar amt | Urd + Cyd, % of radioact. |
|---|---|---|---|---|---|---|
| total RNA | none | 68 | 1.0[b] | 32 | 0.78[b] | nd[f] |
| poly(A)-RNA | pancreatic RNase[c] | 48 | [1.0] | 52 | 1.8 | 0.2 |
| poly(A)-RNA | pancreatic RNase + BAP[d] | 6 | | 4 | | 90 |
| poly(A)-RNA | $T_1$ RNase[e] | 50 | [1.0] | 48 | 1.6 | 1.6 |

[a] Alkaline hydrolysis and analysis of products were as described under Experimental Procedure. [b] Assuming overall base composition of U:C = 26.2:20.4 (average value from Doi and Igarashi, 1964). [c] Incubation with pancreatic ribonuclease (0.2 mg/mL) at 25 °C for 15 min, followed by readsorption to oligo(dT)-cellulose, washing with buffer containing 0.5 M NaCl, and elution with water. [d] Incubation with pancreatic RNase (0.2 mg/mL) and bacterial alkaline phosphatase (BAP; 8 μg/mL) at 25 °C for 15 min, followed by readsorption to oligo(dT)-cellulose, washing with buffer containing 0.5 M NaCl and 1 mM 1,10-phenanthroline (to inactivate alkaline phosphatase), and elution with water. [e] Incubation with $T_1$ RNase (7.7 μg/mL) at 25 °C for 15 min, followed by readsorption to oligo(dT)-cellulose, washing with buffer containing 0.5 M NaCl, and elution with water. [f] Not determined.

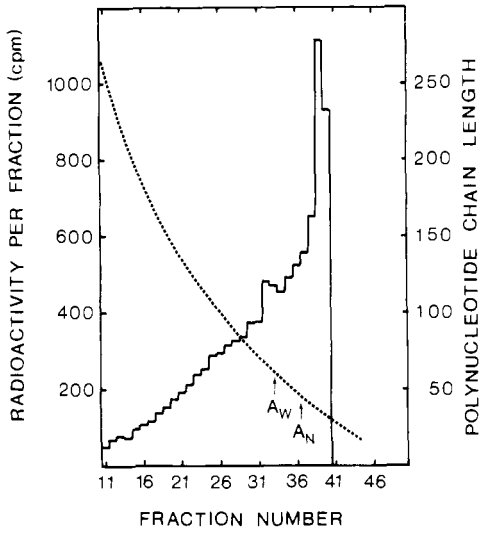


FIGURE 3: Polyacrylamide gel electrophoresis of poly(A) tracts isolated after treatment with pancreatic and $T_1$ RNases. The isolation of poly(A) tracts after digestion with pancreatic and $T_1$ RNases from RNA pulse labeled with [$^3$H]adenosine and the procedure for polyacrylamide gel electrophoresis are described under Experimental Procedure. The *dotted curve* shows the chain length of $(Ap)_nUp$ in each fraction of a polyacrylamide gel run under identical conditions. The *arrows* indicate the weight-average ($A_w$) and the number-average ($A_N$) size of the poly(A) tracts.

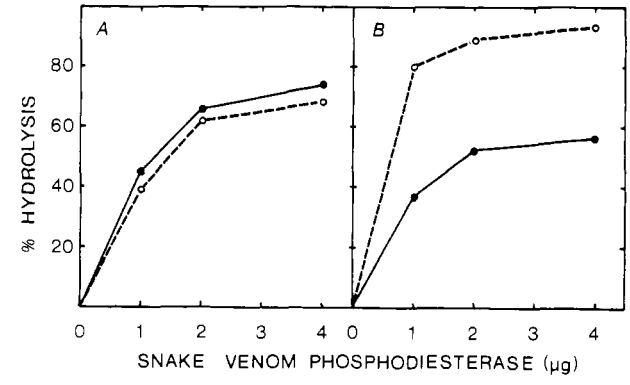


FIGURE 4: Effect of alkaline phosphatase on the degradation by snake venom phosphodiesterase of poly(A) tracts isolated after treatment with pancreatic and/or $T_1$ RNases. Poly(A) tracts were isolated from [$^3$H]-adenosine-labeled RNA after treatment with (A) $T_1$ RNase alone or (B) $T_1$ plus pancreatic RNase, as described under Experimental Procedure. Samples of the poly(A) tracts (about 3500 cpm) were incubated at 40 °C for 1 h in a total volume of 13 μL with 77 mM Tris-HCl, pH 8.5, 7.7 mM $MgCl_2$, and the indicated amounts of snake venom phosphodiesterase, without or with 6 μg of bacterial alkaline phosphatase. After addition of 2 μL of 10 mM 5′-AMP as carrier, samples (10 μL) were applied to a PEI-cellulose sheet and the chromatogram was developed with 1 M LiCl for a distance of 12 cm. The cellulose was scraped off the areas corresponding to adenosine ($R_f$ 0.50), AMP ($R_f$ 0.33), and oligonucleotides ($R_f$ 0) and suspended in 1 mL of 1 M LiCl, and its radioactivity was determined in the scintillation fluid of Anderson and McClure (1973). Percent hydrolysis was calculated from the ratio of radioactivity as adenylate (or adenosine) and oligonucleotide: (●-●) without alkaline phosphatase; (○- - -○) with alkaline phosphatase.

phatase. Figure 4A shows that the exonucleolytic degradation of poly(A) tracts isolated after treatment with $T_1$ RNase was not dependent on prior hydrolysis with alkaline phosphatase. This result suggested the absence of internal guanylate residues between the poly(A) sequence and the 3′ terminus of the RNA molecules. In contrast, when a similar experiment was carried out with poly(A) tracts isolated after treatment with both $T_1$ and pancreatic RNases, only 60% of the poly(A) could be degraded by snake venom phosphodiesterase, the degradation of the remainder requiring prior treatment with alkaline phosphatase (Figure 4B). This indicated that in 60% of the RNA chains the poly(A) sequence was either at the 3′ end or directly adjacent to the 3′ terminal residue, while in 40% of the chains at least one internal pyrimidine nucleotide residue intervened between the poly(A) tract and the 3′ terminus.

*Nucleotides at the 3′ End of the Poly(A) Sequences.* If a significant fraction of the poly(A) tracts were indeed located at a 3′-hydroxyl terminus of the RNA molecules, alkaline hydrolysis should release the terminal residue as adenosine, while the internal residues should yield 2′(3′)-AMP. Poly(A) sequences were isolated from RNA pulse labeled with [$^3$H]-adenosine by adsorption to oligo(dT)-cellulose, followed by digestion with pancreatic RNase and subsequent elution at low ionic strength. The products of alkaline hydrolysis were separated by electrophoresis in borate buffer at pH 9.2. In a typical experiment, 39 500 cpm was found to be associated with 2′(3′)AMP and 236 cpm with adenosine. Comparable results were obtained when the two components were separated by chromatography on thin layers of PEI-cellulose. This suggested that $^1/_{167}$ of the adenylate residues in the poly(A) fraction obtained by digestion with pancreatic RNase was 3′ terminal. It should be noted in regard to this experiment that no radioactivity appeared in guanylate residues after pulse labeling with [$^3$H]adenosine, so that the radioactivity provided a specific measure of adenylate residues.

The nature of the nucleotide adjacent to adenylate in those chains in which the poly(A) sequence is not at the 3′ terminus was examined by alkaline hydrolysis of RNA labeled with [$^3$H]uridine (Table II). Analysis of the alkaline hydrolysate of total pulse-labeled RNA by chromatography on thin layers

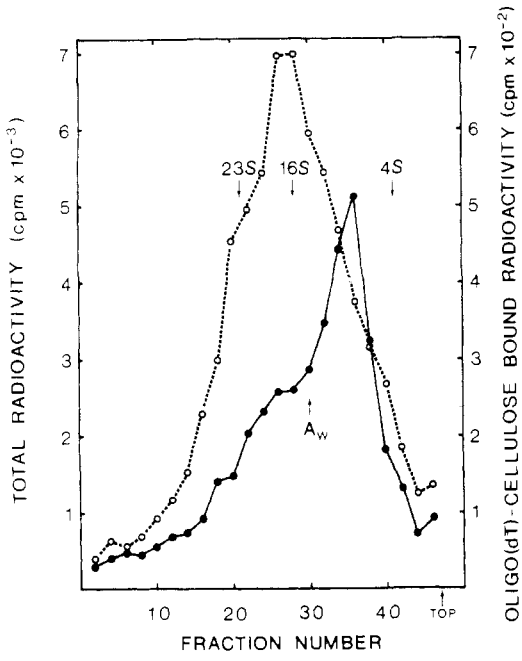


FIGURE 5: Sucrose gradient centrifugation of pulse-labeled RNA. RNA, pulse labeled with [$^3$H]uridine, was subjected to sucrose gradient centrifugation and analysis for radioactivity in (O- - -O) total RNA and in (●-●) RNA containing poly(A) sequences as described under Experimental Procedure. The *arrows* indicate the positions of sedimentation of tRNA (4 S) and of rRNA (16 and 23 S). $A_w$ denotes the weight average position of sedimentation of poly(A)-RNA.

of PEI-cellulose showed that about two-thirds of the radioactivity was associated with 2′(3′)UMP and one-third with 2′-(3′)CMP. Assuming the average nucleotide composition of pulse-labeled RNA from *B. brevis* to be similar to that from *B. subtilis* as determined by Doi and Igarashi (1964), the relative specific activities of UMP and CMP were 1.0 and 0.60, respectively, in RNA pulse labeled with [$^3$H]uridine. A similar analysis of the poly(A) tracts isolated by elution from oligo(dT)-cellulose at low ionic strength after digestion with pancreatic RNase revealed about equal amounts of radioactivity in UMP and CMP and none in the nucleoside fraction. Accordingly, the pyrimidine nucleotides adjacent to the 3′ end of the poly(A) sequences were exclusively internal UMP and CMP residues in a molar ratio of 1 to 1.8. The fact that 90% of these residues was released as nucleosides after treatment of the pancreatic RNase fragment with bacterial alkaline phosphatase confirmed their position at the 3′ end of the poly(A) sequences. A similar ratio of UMP and CMP and the absence of uridine and cytidine were also found in the alkaline hydrolysates of poly(A)-containing sequences obtained after treatment with $T_1$ RNase. Since there is no cleavage site for $T_1$ RNase between the poly(A) sequences and the 3′ ends of the RNA molecules (see above), the absence of pyrimidine nucleosides from the hydrolysate established that the poly(A)-containing RNA molecules had no 3′-hydroxyl terminal pyrimidine nucleotide residues.

*Characterization of Poly(A)-Containing RNA.* Sucrose gradient sedimentation of total RNA pulse labeled with [$^3$H]uridine revealed a broad peak with a midpoint at 16 S (Figure 5). Analysis of each fraction for poly(A)-containing RNA by adsorption to oligo(dT)-cellulose showed a polydisperse sedimentation pattern, with a peak at 7 S and a broad shoulder of more rapidly sedimenting material. The weight-average sedimentation coefficient of the poly(A)-containing RNA was 12.5 S, corresponding to an average length of 800–900 nucleotide residues (Boedtker, 1968).

The high poly(A) content was characteristic of pulse-labeled RNA. In an experiment in which cells of *B. brevis* were labeled for 30 s with [$^3$H]adenosine, the poly(A) content was found to be 38%. This value was reduced to 11% when the labeling was followed by a 30-min incubation with unlabeled adenosine.

## Discussion

The results presented here show that a substantial fraction (30–40%) of pulse-labeled RNA from *B. brevis* contains poly(A) sequences. This finding is of considerable interest because such a high content of poly(A)-RNA had been considered typical of eukaryotic systems and had not been previously reported in prokaryotes. In fact, poly(A)-containing RNA has been described in only two prokaryotic species—*Escherichia coli*, where it constituted between 0.2 (Nakazato et al., 1975) and 15% (Srinivasan et al., 1975) of pulse-labeled RNA, depending upon growth conditions, and in *Caulobacter crescentus*, where it accounted for 7.6% of rapidly labeled RNA (Ohta et al., 1975). The weight-average length of the poly(A) tracts in pulse-labeled RNA from *B. brevis* was 60 nucleotide residues. The size range extended from 30 to 260 nucleotides, but 75% of the molecules was distributed in the relatively narrow range of 50 ± 14 residues. This is a somewhat shorter average length than that found in mammalian mRNA (100–170 residues; Brawerman, 1974), in *Dictyostelium discoideum* (100 residues; Jacobson et al., 1974), and in *Tetrahymena pyriformis* (80–150 residues; Rodriguez-Pousada and Hayes, 1976), but in the same range as that in yeast mRNA (50 residues; McLaughlin et al., 1973), in *Neurospora crassa* (30–70 residues; Lucas et al., 1977), in mammalian mitochondria (50–80 residues; Perlman et al., 1973), and in *E. coli* (45 residues; Nakazato et al., 1975; or 4 S, Srinivasan et al., 1975), and longer than that found in *Caulobacter crescentus* (13–17 residues; Ohta et al., 1978).

The higher poly(A) content of pulse-labeled RNA from *B. brevis* compared to that in RNA from other prokaryotes might, of course, be due to a difference in isolation methods. Our procedure involved immediate lysis and proteinase K digestion of pulse-labeled cells, conditions under which ribonuclease action should be minimized (Wiegers and Hilz, 1971; Morrison et al., 1977), followed directly by adsorption to oligo(dT)-cellulose. This contrasts with other methods that generally involve a phenol-extraction step in which mechanical loss and RNA degradation are difficult to control. On the other hand, it is possible that the unusually high poly(A) content may be typical of *Bacillus* species. The presence of poly(A)-containing RNA has recently been also reported in *Bacillus subtilis* (Graef and Chambliss, 1978).

The location of the poly(A) tracts in the RNA molecules was deduced from the susceptibility to degradation by snake venom phosphodiesterase after preliminary treatment with either $T_1$ or pancreatic RNase. The results showed that the action of $T_1$ RNase did not generate 3′-phosphoryl end groups on the RNA fragments containing the poly(A) tracts, thus excluding the occurrence of internal guanylate residues between the poly(A) sequence and the 3′ terminus of the molecule. On the other hand, 40% of the poly(A)-containing fragments generated by the action of pancreatic RNase had 3′-phosphoryl end groups, indicative of one or more internal pyrimidine nucleotide residues between the poly(A) sequence and the 3′ terminus. It appears, therefore, that pulse-labeled RNA from *B. brevis* contains two classes of poly(A) sequences. In one class, which represents 60% of the poly(A), the poly(A) sequence is directly adjacent to the 3′-terminal residue of the RNA chain, whereas

in the other class, accounting for 40% of the poly(A), at least one internal pyrimidine nucleotide residue (but no guanylate) intervenes between the poly(A) sequence and the 3′ terminus of the molecule.[3]

A more difficult problem is the identification of the nucleotide residues at the 3′ end of the poly(A)-containing RNA molecules. Since the RNA of interest is defined by pulse-labeling and is not physically separated from other RNA species that may have different poly(A) contents or terminal sequences, it is not possible to apply chemical labeling procedures to identify the 3′-terminal residues. Rather, the characterization of the 3′ ends must make exclusive use of the radioactivity introduced by pulse labeling. The ratio of internal to 3′-hydroxyl terminal adenylate residues in poly(A)-containing fragments isolated after treatment of [$^3$H]adenosine-labeled RNA with pancreatic RNase was found to be 167. Since 40% of the material produced after pancreatic RNase digestion is known to be terminated with pyrimidines (see above), the 3′-terminal adenylate residues must be associated with the remaining 60% of the poly(A) tracts. Accordingly, the pyrimidine-free poly(A) fragments generated by pancreatic RNase must have a ratio of internal to 3′-terminal adenylate residues of about 100. This is consistent with either of the two limiting patterns of distribution of molecular species illustrated in Figure 6. (1) If both the pyrimidine-terminated and the other RNA chains have the same size distribution (weight-average length of 60 nucleotides), at most two-thirds of the latter (40% of the total chains) can be terminated with adenosine. The remaining 20% of the molecules must thus have a 3′-terminal guanosine moiety.[4] (2)Alternatively, the size distribution of pyrimidine-terminated and other RNA species may be different. If the former constituted all the shorter chains seen on electrophoresis (Figure 3), with a weight-average length of 40, the remaining 60% of the material would have an average length of 90 residues and could therefore be all terminated with adenosine.

The nature of the 40% of the 3′-terminal sequences of the poly(A)-containing RNA chains in which a pyrimidine nucleotide is adjacent to the poly(A) tract was studied by alkaline hydrolysis of the poly(A)-containing fragments generated by pancreatic or $T_1$ RNase digestion of [$^3$H]uridine-labeled RNA, in which uridylate and cytidylate residues were labeled in the ratio of 1.0:0.6. The pyrimidine nucleotides in the alkaline hydrolysate of poly(A) segments produced by pancreatic RNase treatment consisted of 65% CMP and 35% UMP on a molar basis and must represent the residues directly adjacent to the poly(A) sequence. A similar ratio of CMP to UMP was found in the alkaline hydrolysate of poly(A) segments after digestion with $T_1$ RNase, indicating that, if there are more than one pyrimidine nucleotide residue at the 3′ end of the poly(A) sequences, they must be present in nearly the same proportion. No significant amounts of pyrimidine nucleosides were seen in the alkaline hydrolysates of either kind of RNase digest,

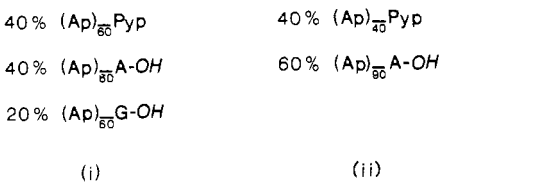


FIGURE 6: Possible patterns of distribution of polynucleotide chains in pancreatic RNase digest of poly(A)-RNA. For explanation, see text.

suggesting that none of the poly(A)-containing RNA molecules had 3′-hydroxyl terminal pyrimidine nucleotide residues. The identity of the terminating moiety in this class of RNA chains has not been further investigated.

It is clear from the results described here that the poly(A) sequences in pulse-labeled RNA from *B. brevis* are located near the 3′ ends of the molecules but that the terminal oligonucleotide sequence is somewhat heterogenous. The general position of the poly(A) tracts thus resembles that found in poly(A)-RNA molecules of both prokaryotes and eukaryotes. Whether the limited heterogeneity at the 3′ terminus is unique to poly(A)-containing RNA from *B. brevis* is a question which cannot be answered at this time, since the 3′-terminal sequences in poly(A)-RNA from other prokaryotes have not been analyzed at the same level of resolution. However, the composition of the 3′ termini of some eukaryotic poly(A)-RNAs has been studied in sufficient detail to establish that the poly(A) sequence is directly at the 3′-end (Nakazato et al., 1973; Molloy and Darnell, 1973; McLaughlin et al., 1973). The presence of at least some residues other than adenylate at the 3′ ends of the poly(A) tracts in *B. brevis* may have implications both on the mode of processing and the function of these RNA molecules.

The nature of the RNA molecules containing poly(A) sequences was examined by sucrose gradient sedimentation. Pulse-labeled poly(A)-RNA sedimented as a skewed peak with a weight-average sedimentation coefficient of 12.5 S, corresponding to a polynucleotide chain length of 800–900 residues. This size distribution differs from that of any of the known stable RNA species but is consistent with that expected for mRNA. Indeed, the observation that pulse-labeled RNA contains a significantly higher proportion of poly(A)-RNA than long-term-labeled RNA suggests that poly(A)-RNA turns over relatively rapidly, in agreement with the observations on *Caulobacter crescentus* (Ohta et al., 1975, 1978) and *E. coli* (Nakazato et al., 1975). In *B. subtilis*, poly(A)-containing RNA has actually been found to have mRNA activity (Graef and Chambliss, 1978).

Little can be said at this time about the possible function of poly(A) sequences in bacterial mRNA, especially since the mode of synthesis of these sequences has not yet been investigated. For example, it is not clear whether poly(A) is transcribed from poly(T) stretches in the DNA or whether it is added after transcription. However, it is interesting that *B. brevis* contains an exonuclease which is subject to allosteric regulation by guanosine 3′,5′-monophosphate (Sarkar and Paulus, 1975). This enzyme degrades RNA from the 3′ end but acts very slowly on poly(adenylic acid). It has been shown that the enzyme is responsible for the rapid degradation of newly synthesized RNA in toluene-treated cells of *B. brevis* and that RNA synthesized by sporulating cells is more vulnerable to degradation than RNA from exponentially growing cells (Paulus and Sarkar, 1974). It is thus not implausible that the degradation of mRNA in *B. brevis* might be controlled by the addition of 3′-terminal poly(A) sequences at specific stages during the growth cycle. However, a careful comparison of the

[3] The possibility might be considered that both classes of poly(A) sequences might exist in the same molecule, a situation that would arise if one or more pyrimidine nucleotide residues were interspersed in a longer poly(A) sequence. However, this is ruled out by the observation that the size distribution of the poly(A) tracts generated by treatment with $T_1$ RNase is indistinguishable from that seen after treatment with both pancreatic and $T_1$ RNase.

[4] The possibility that some chains may be terminated by a 3′-phosphate is eliminated by the results in Figure 4A which show that exonucleolytic degradation of poly(A) tracts generated by $T_1$ RNase is not affected by prior treatment with bacterial alkaline phosphatase. The results to be discussed below eliminate the presence of terminal pyrimidine nucleoside moieties.

poly(A) content of vegetative and sporulating mRNA has yet to be carried out.

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# Identification of the Transcribing DNA Strand for the Deoxynucleotide Kinase Gene of Bacteriophage T2†

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ABSTRACT: A modified procedure was developed which allows RNA–DNA hybridization reactions to be performed without the loss in translational capacity of mRNA which accompanies hybridization at elevated temperatures or in the presence of the denaturing agent formamide. Separated l and r strands of bacteriophage T2 DNA were hybridized in the presence of 4 M sodium perchlorate at 37 °C with total RNA from infected cells. After passage of the hybridization mixture through a nitrocellulose column to remove single-strand DNA and DNA–RNA hybrids, the eluent was measured for its capacity to promote deoxynucleotide kinase (gene 1) synthesis in an in vitro protein-synthesizing system derived from uninfected *Escherichia coli.* With this procedure, which should be of general use for any message whose product can be measured either enzymatically, immunologically, or by location in an acrylamide gel, it was demonstrated that deoxynucleotide kinase mRNA is transcribed from the l strand of bacteriophage T2 DNA. By titrating with l strand DNA, the number of deoxynucleotide kinase transcripts present 9 min after T2 phage infection at 30 °C was estimated to be about 38 copies per cell.

RNA–DNA hybridization techniques and cell-free translation systems have added immeasurably to our knowledge about the synthesis and regulation of specific mRNAs in numerous biological systems. Each methodology, however, possesses a limited capacity to provide information about a given mRNA. For example, translational assays can indicate the presence or absence of a specific mRNA, providing an assay for its product is available, but it cannot reveal more than a qualitative estimate of the abundance of the mRNA. Hybridization methods can provide an accurate measure of mRNA abundance, but this may require either the isolation of nonlethal mutants deficient in the gene transcript or the laborious purification of the mRNA and preparation of its DNA complement to serve as a probe.

By coupling the strengths of both cell-free translation assays and hybridization techniques, we have developed a system for

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0006-2960/78/0417-3474$01.00/0 © 1978 American Chemical Society