Characterization of Polyadenylate-Containing Ribonucleic Acid from *Bacillus subtilis*†

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ABSTRACT: Affinity chromatography of pulse-labeled RNA from *Bacillus subtilis* on oligo(dT)-cellulose or poly(U)-agarose revealed the presence of 10-20% polyadenylate-containing RNA. The poly(A) tracts were isolated after digestion with pancreatic and T_1 ribonucleases and represented about 4% of the poly(A)-RNA; their weight-average length was 49 nucleotide residues. Of the poly(A) tracts 60-75% had free 3'-hydroxyl termini and must thus have been located directly at the 3' ends of the poly(A)-RNA. The poly(A) content of unlabeled RNA of *B. subtilis* was measured by its ability to form ribonuclease-resistant hybrids with [^{3}H]poly(U). Poly(A) sequences were found to constitute 3.9% of poly(A)-RNA, which agrees closely with the poly(A) content of pulse-labeled RNA calculated from its ribonuclease resistance. The poly(A) content of total *B. subtilis* RNA was found to be 0.091%, indicating that the steady-state level of poly(A)-RNA in *B. subtilis* is 2.3% of the total RNA or approximately 25% of the mRNA. The mRNA activity of poly(A)-RNA and of total *B. subtilis* RNA was compared by using a cell-free translation system from *Escherichia coli*. Poly(A)-RNA was 8 times more effective than total RNA in stimulating protein synthesis, indicating a considerable enrichment of mRNA in this fraction. The sequence complexity of poly(A)-RNA was examined by hybridization with restriction endonuclease generated fragments of *B. subtilis* DNA, and complementarity to many different DNA sequences was found. Pulse-labeled poly(A)-RNA had a high turnover rate, but its level was relatively constant at all stages of growth and sporulation and was not significantly different in mutants blocked at stage O of sporulation. These observations indicate that poly(A)-RNA is functional mRNA, that it represents a significant proportion of *B. subtilis* mRNA, and that it functions during both growth and sporulation.

In the past few years, much evidence has accumulated to suggest that polyadenylate sequences are a characteristic feature of bacterial as well as of eucaryotic mRNA (Nakazato et al., 1975; Srinivasan et al., 1975; Ohta et al., 1975, 1978; Sarkar et al., 1978; Graef-Dodds & Chambliss, 1978; Schultz et al., 1978; Kaur & Jayaraman, 1979; Kerjan & Szulmajster, 1980; Hecker et al., 1981; Gopalakrishna et al., 1981). Many of the earlier reports on polyadenylated bacterial RNA described relatively low or variable levels, probably due to specific losses of poly(A)-RNA in the course of conventional RNA isolation procedures. However, using a new RNA isolation procedure involving proteinase K, we have been able to demonstrate consistently high levels of poly(A)-RNA in the pulse-labeled RNA fraction of *Bacillus brevis*, *Bacillus subtilis*, and *Escherichia coli* (Gopalakrishna et al., 1981) and have characterized the polyadenylated RNA of *B. brevis* in considerable detail (Sarkar et al., 1978). In this paper, we extend our studies of polyadenylated RNA to *B. subtilis*, an organism that, unlike *B. brevis*, has been the subject of detailed biochemical and genetic analysis and thus provides a more appropriate context for the analysis of poly(A)-RNA function. Besides characterizing some aspects of the structure of pulse-labeled poly(A)-RNA, we also measure the steady-state levels of poly(A) sequences in *B. subtilis* RNA and present data that are relevant to the function of poly(A)-RNA, such as its mRNA activity, its sequence complexity, and its synthesis at various stages during growth and sporulation.

Experimental Procedures

Materials. Oligo(dT)-cellulose (Type 7) was obtained from P-L Biochemicals, poly(U)-agarose was from Pharmacia, [2,8-^{3}H]adenosine (25.5 Ci/mmol) was from ICN, [5-^{3}H]-uridine (25 Ci/mmol), [^{3}H]poly(uridylic acid) (5.2 Ci/mmol), L-[3,4,5-^{3}H]leucine (110 Ci/mmol), and [^{32}P]P_i (carrier free) were from New England Nuclear, agarose (type I) was from Sigma, and Macaloid was from National Lead Co. Proteinase K (Boehringer) was incubated for 20 min at 37 °C (5 mg/mL in 10 mM Tris,[1] pH 7.5, containing 0.5% sodium dodecyl sulfate) just prior to use. The sources of all other materials were described earlier (Sarkar et al., 1978; Gopalakrishna et al., 1981).

Bacterial Strains and Growth Conditions. B. subtilis ATCC 23856 was grown in nutrient broth supplemented with salts and glucose (Hanson et al., 1964) on a rotary shaker at 37 °C, and growth was followed with a Klett-Summerson colorimeter by using a no. 42 filter. Other *B. subtilis* strains, 1A1 (spo$^+$ trpC2), 1A96 (spo$^+$ trpC2 pheA1), 1S10 (spo OA12 trpC2), 1S11 (spo OA12 trpC2 to1B24), and 1S27 (spo OJ37 metC3 tal-1), were obtained from the Bacillus Genetic Stock Center, Ohio. *E. coli* MZ9 was a gift from Dr. A. Torriani and was grown in YK medium (Hopper et al., 1975).

Labeling of RNA. Pulse labeling was done for 30 s with [2,8-^{3}H]adenosine (25 Ci/mmol) by using 10 μCi/mL for vegetative cells below 200 Klett units, 15 μCi/mL for cells between 200 and 300 Klett units, and 30 μCi/mL for later stages. Pulse labeling with [^{32}P]P_i was done for 5 min in nutrient broth that had been partially depleted of P_i by precipitation with an equivalent amount of $CaCl_2$ at a nutrient broth concentration of 0.5 g/mL. The pattern of growth and sporulation was the same as in standard medium. The pulse-labeled cells were processed as described earlier (Gopalakrishna et al., 1981).

Isolation of RNA. Pulse-labeled poly(A)-RNA was isolated as described previously (Sarkar et al., 1978) by batchwise binding of the proteinase K lysate to oligo(dT)-cellulose, except that the adsorption step was 2 h at 2 °C. Unlabeled RNA

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; AMP, adenosine phosphate.

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was isolated by a different procedure, because traces of proteinase K might have interfered with some of its uses. The bacterial cultures were treated with 25 mM NaN_3 and chilled rapidly, and the cells were collected by centrifugation at 20000*g* for 20 min. The cells were suspended in 0.01 culture volume of a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 10 mM NaN_3, 7 mg/mL Macaloid, and 1% sodium dodecyl sulfate, mixed with an equal volume of water-saturated phenol, and disrupted in a French pressure cell. Further processing was as described by Chambliss & Legault-Demare (1977). The total RNA was then fractionated into poly(A)-RNA and unadenylated RNA by batchwise adsorption to oligo(dT)–cellulose at 2 °C as described earlier (Sarkar et al., 1978), except that KCl was used instead of NaCl. *E. coli* B tRNA (Schwarz/Mann) was added as carrier prior to precipitation of the poly(A)-RNA fraction. Ribosomal RNA was isolated by phenol extraction from ribosomes that had been freed from extraneous RNA fractions by dialysis against a buffer containing 0.1 mM $MgCl_2$ and centrifugation at 100000*g* for 6 h.

Isolation of Poly(A) Tracts by Nuclease Digestion. Cultures (2 mL) were pulse labeled with [^{3}H]adenosine (25 μCi/mL) and processed with proteinase K as described above. The lysate was absorbed to oligo(dT)–cellulose (30 mg) and washed with high-salt buffer. The oligo(dT)–cellulose containing bound poly(A)-RNA was suspended in 1.0 mL of Tris-HCl, pH 7.5, containing 0.5 M NaCl and 2 mM EDTA and was digested with 30 μg of ribonuclease A and 90 units of T_1 for 30 min at 25 °C. Under these conditions, 99.9% of [^{3}H]uridine-labeled poly(A)-RNA absorbed to oligo(dT)–cellulose was rendered acid soluble. After digestion, poly(A) tracts were eluted with water and recovered as described by Sarkar et al. (1978).

Digestion of Isolated Poly(A) Tracts with Snake Venom Phosphodiesterase. Poly(A) tracts (2500 cpm), isolated as described above, were incubated at 40 °C for 90 min in a final volume of 20 μL with 20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, and 6 μg of snake venom phosphodiesterase without or with 12 μg of bacterial alkaline phosphatase. The release of 5′-AMP was measured by thin-layer chromatography as described by Sarkar et al. (1978).

Analytical Methods. The estimation of polyadenylate chain length by electrophoresis on polyacrylamide gels, the thin-layer chromatographic separation of 5′-AMP and oligonucleotides, and the thin-layer electrophoretic separation of adenosine and 2′(3′)-AMP have been described previously (Sarkar et al., 1978). RNA was estimated by the orcinol method (Schneider, 1957).

Hybridization of [^{3}H]Poly(U) with RNA. The poly(A) of unlabeled RNA preparations was assayed by annealing with [^{3}H]poly(U) (5 Ci/mmol). Various RNA fractions were incubated in a total volume of 50 μL with 10 nCi of [^{3}H]-poly(U) in 10 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM $MgCl_2$, and 0.2% (w/v) sodium dodecyl sulfate for 15 min at 25 °C. Further processing is as described by Bergmann & Brawerman (1980) except 1.25 μg/mL pancreatic RNase was used and RNase-resistant poly(A)·poly(U) hybrids were precipitated with 2.5% trichloroacetic acid for only 10 min to prevent loss of polymeric radioactivity, which is dependent on both acid concentration and the time of exposure to acid (Williams & Klett, 1978).

Cell-Free Protein Synthesis. E. coli MZ9 was grown, harvested, and washed as described previously (Hagen & Young, 1973; Hopper et al., 1975). All subsequent steps were done at 4 °C. The cells were suspended immediately (1.5 mL/g wet weight) in buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, and 10 mM magnesium acetate and were passed through a French pressure cell at 20000 psi. The lysate was supplemented with 2-mercaptoethanol (6 mM) and centrifuged for 30 min at 30000*g*. The supernatant solution was stored in small portions at −80 °C. The incorporation of [^{3}H]leucine into protein was assayed under the conditions described by Hagen & Young (1973) except that the reaction mixture contained 0.2 mM dithiothreitol. Prior to assay, the *E. coli* supernatant fraction was incubated for 15 min at 37 °C in half the final volume of a mixture containing all assay components except [^{3}H]leucine and mRNA, in order to reduce the endogenous level of protein synthesis. The actual assay was carried out for 30 min at 37 °C, the reaction was terminated by the addition of 0.3 N trichloroacetic acid, and the acid-insoluble material remaining after 15 min at 100 °C was collected by filtration on glass-fiber filters and its radioactivity determined in a liquid scintillation counter.

Hybridization of [^{32}P]RNA to DNA. DNA was isolated from *B. subtilis* by the gentle procedure of Harris-Warrick et al. (1975) and further purified by centrifugation in CsCl gradients. Restriction endonuclease *Eco*RI generated fragments were electrophoretically separated in 0.7% agarose gels and transferred to nitrocellulose membranes as described by Southern (1975). Hybridization of ^{32}P-labeled poly(A)-RNA and nonpoly(A)-RNA, isolated by the proteinase K method, was done at 68 °C for 18 h with nitrocellulose strips containing excess of immobilized DNA restriction fragments by using incubation conditions, washing procedures, and autoradiography as described by Birg et al. (1977). Hybridization appeared nearly complete in 18 h, longer incubation (up to 72 h) having no effect on the intensity or number of bands observed.

Results

Poly(A)-RNA Content of Pulse-Labeled RNA. When pulse-labeled RNA from *B. subtilis* was analyzed for polyadenylate sequences by affinity chromatography on oligo(dT)–cellulose, between 10 and 20% was bound at high ionic strength and eluted at low-salt concentrations, regardless of the labeled precursor used (Table I). About 14% of the poly(A)-RNA isolated after labeling with [^{3}H]adenosine was resistant to exhaustive digestion by a mixture of T_1 and pancreatic ribonucleases and presumably represented poly(A) tracts, no ribonuclease-resistant material being found when the RNA had been labeled with [^{3}H]uridine. Similar results were obtained with affinity chromatography on poly(U)–agarose. On the other hand, very little labeled poly(A)-RNA was found when pulse labeling with [^{3}H]adenosine was followed by a 1-h period of incubation with unlabeled adenosine.

Length of the Poly(A) Sequences. The size distribution of the poly(A) tracts obtained by the digestion of pulse-labeled poly(A)-RNA with a mixture of ribonucleases A and T_1 was examined by polyacrylamide gel electrophoresis. As shown in Figure 1, the poly(A) sequences migrated as a relatively sharp peak that was followed by polydisperse higher molecular weight material. The weight-average chain length was 49 nucleotide residues and the number-average chain length 37 residues, 70% of the material being in the length range of 35–80 nucleotide residues.

Location of the Poly(A) Sequences in the RNA Chains. Two procedures were used to determine whether the poly(A) tracts are located at the 3′ end of poly(A)-RNA, both being based on the fact that such a location would lead to the production of 3′-hydroxyl terminated poly(A) sequences after treatment with a mixture of ribonucleases A and T_1. One

Table I: Characteristics of Pulse-Labeled Poly(A)-RNA[a]

RNA sample	radioactivity eluted at low salt: cpm	% of input	% ribonuclease resistant
RNA labeled 30 s with [^{3}H]Ado	6.7×10^4	10.3	13.5
RNA labeled 30 s with [^{3}H]Urd	3.4×10^3	20.0	<0.1
RNA labeled 5 min with [^{32}P]P_i	6.0×10^6	10.9	nd[b]
RNA labeled 30 s with [^{3}H]Ado, then incubated 60 min with unlabeled Ado	500	0.2	nd
RNA labeled 30 s with [^{3}H]Ado[c]	1.1×10^4	15.6	nd

[a] Pulse-labeled RNA was isolated and fractionated on oligo(dT)-cellulose as described under Experimental Procedures. A duplicate sample, while still adsorbed to oligo(dT)-cellulose, was treated with a mixture of pancreatic and T_1 ribonucleases as described under Experimental Procedures and washed, and the ribonuclease-resistant material was then eluted at low ionic strength. [b] nd, not determined. [c] The sample was fractionated on poly(U)-agarose.

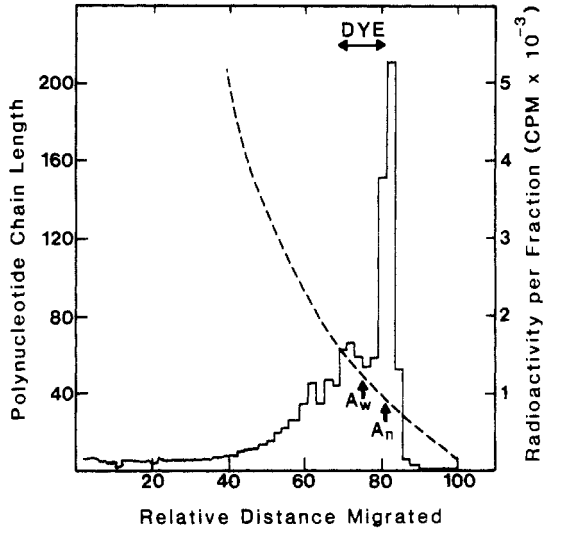

FIGURE 1: Polyacrylamide gel electrophoresis of poly(A) tracts isolated from poly(A)-RNA. The isolation of poly(A) tracts after digestion with pancreatic and T_1 RNases from RNA pulse labeled with [^{3}H]adenosine and the procedure for polyacrylamide gel electrophoresis are described under Experimental Procedures. The dashed curve shows the chain lengths of the synthetic polymer $(Ap)_nU_p$ (n = 70, range = 15–250), labeled with [^{14}C]adenosine and [^{3}H]uridine, which was run in a parallel gel. The arrows indicate the weight-average (A_w) and the number-average (A_n) chain length of the poly(A) tracts.

approach involved the alkaline hydrolysis of [^{3}H]adenosine-labeled poly(A) tracts, followed by the estimation of the relative amounts of [^{3}H]adenosine and [^{3}H]-2′(3′)-AMP, separated by paper electrophoresis in a borate buffer. A typical experiment showed 17 190 cpm associated with 2′(3′)-AMP and 263 cpm with adenosine, corresponding to internal and 3′-hydroxyl terminal residues, respectively. If all poly(A) sequences, with a weight-average length of 49 residues, had been directly at the 3′ terminus of the RNA, the amount of radioactivity expected in the adenosine fraction would have been 17 190/49 or 350 cpm. Our results thus indicate that 75% of the poly(A) sequences were located directly at the 3′ terminus, whereas the remaining 25% were separated from the 3′ end by at least one internal pyrimidine or guanine nucleotide residue.

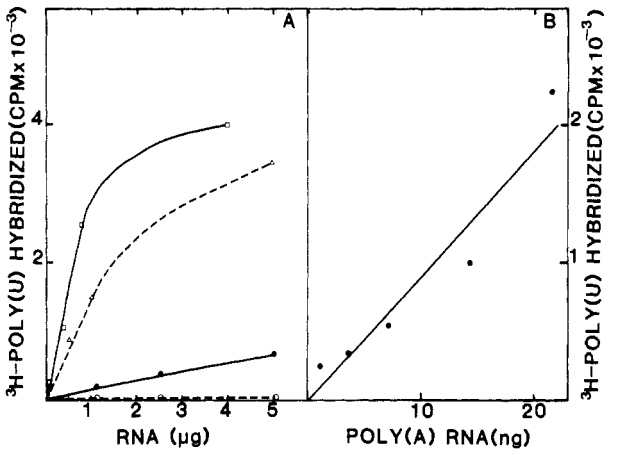

FIGURE 2: Hybridization of [^{3}H]poly(U) with various RNA fractions from *B. subtilis*. Total RNA, poly(A)-RNA, nonpoly(A)-RNA, and rRNA were isolated from *B. subtilis* cells, and hybridization of various concentrations of RNA with [^{3}H]poly(U) was done as described under Experimental Procedures. (A) Total RNA isolated by the proteinase K method (□), total RNA isolated by phenol extraction (△), nonpoly(A)-RNA (●), and ribosomal RNA (○). Standard curve with poly(adenylic acid) gave a value of 3120 cpm/ng. (B) Poly(A)-RNA isolated from total RNA by using the proteinase K method (●). Standard curve with poly(adenylic acid) gave a value of 2300 cpm/ng.

The other approach measured the susceptibility of [^{3}H]-adenosine-labeled poly(A) tracts, obtained by digestion with ribonucleases A and T_1, to hydrolysis by snake venom phosphodiesterase, an exonuclease specific for polynucleotides with a free 3′-hydroxyl terminus. It was found that only 50–65% of the poly(A) sequences could be degraded by snake venom phosphodiesterase, whereas 80–95% hydrolysis was seen if the poly(A) tracts were first treated with alkaline phosphatase, suggesting that 30–40% of the poly(A) sequences were originally not directly at the 3′ terminus of the RNA molecules.

Poly(A) Content of Total Cellular RNA. In order to obtain an independent confirmation of the presence of poly(A)-RNA in *B. subtilis* as well as a measure of the actual steady-state level in total cellular RNA, we examined the ability of unlabeled *B. subtilis* RNA to form a ribonuclease-resistant hybrid with ^{3}H-labeled poly(uridylic acid) (Bergmann & Brawerman, 1980). Bulk unlabeled RNA was isolated from *B. subtilis* by a modification of the phenol extraction procedure of Chambliss & Legault-Demare (1977) or with our proteinase K procedure (Gopalakrishna et al., 1981) and annealed with [^{3}H]poly(U). As shown in Figure 2, the amount of [^{3}H]poly(U) protected from degradation by pancreatic ribonuclease was a function of the amount of RNA present in the hybridization mixture. By using known amounts of poly(adenylic acid) as a standard, it could be calculated that 1 μg of total RNA from *B. subtilis* contained 0.91 ng of poly(A) sequences if isolated by the proteinase K procedure or 0.59 ng of poly(A) if obtained by phenol extraction (Figure 2A). No significant levels of polyadenylate could be detected in ribosomal RNA and in the RNA fraction not adsorbed to oligo(dT)–cellulose at high ionic strength, whereas RNA that bound to oligo(dT)–cellulose at high salt and was eluted by water was greatly enriched in poly(A) sequences, containing 39 ng of poly(A)/μg of RNA (Figure 2B).

Messenger Activity of Poly(A)-RNA. In an attempt to define the function of poly(A)-RNA in *B. subtilis*, we compared the ability of different RNA fractions to serve as a template for protein synthesis in the cell-free system from *E. coli* MZ9 developed by Arnaud et al. (1980). The incoporation of [^{3}H]leucine into acid-precipitable protein was stimulated between 10- and 20-fold by the addition of *B. subtilis* RNA,

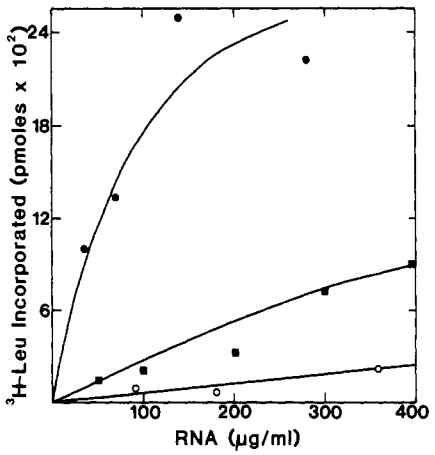

FIGURE 3: Effect of RNA concentration on [^{3}H]leucine incorporation into protein in an *E. coli* cell-free system by using various types of RNA from *B. subtilis*. Total RNA, poly(A)-RNA, and nonpoly(A)-RNA were isolated, messenger activity of these RNA fractions was assayed by using *E. coli* MZ9 extract, and [^{3}H]leucine incorporation into protein was measured as described under Experimental Procedures. Poly(A)-RNA (●), total RNA (■), and nonpoly(A)-RNA (○).

Table II: Proportion of Pulse-Labeled Poly(A)-RNA at Different Stages of Growth and Sporulation[a]

growth stage	% poly(A)-RNA
mid-exponential (100 Klett units)	15
late exponential (200 Klett units)	10
sporulating, $t_{0.5}$ (300 Klett units)[b]	12
sporulating, t_2	9
sporulating, t_4	12

[a] Pulse labeling with [^{3}H]adenosine, cell lysis, and fractionation of RNA on oligo(dT)–cellulose were as described under Experimental Procedures. [b] $t_{0.5}$, 0.5 h after the end of exponential growth.

different RNA fractions varying in their effectiveness. As shown in Figure 3, poly(A)-RNA was about 8 times more effective in stimulating [^{3}H]leucine incorporation than unfractionated RNA, whereas the RNA fraction that was not bound to oligo(dT)–cellulose at high ionic strength was relatively inactive. Preliminary experiments showed that upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, most of the radioactive material had a molecular weight in the 5000–10 000 range, but significant amounts of radioactivity were also found as discrete bands in the 20 000–45 000 molecular weight range, with no significant differences in the banding pattern obtained with total RNA or poly(A)-RNA as the template (data not shown).

Developmental Regulation of Poly(A)-RNA. The effect of the developmental stage on the synthesis of poly(A)-RNA was examined by pulse labeling cultures of *B. subtilis* with [^{3}H]adenosine at various stages of growth and sporulation and analyzing the labeled RNA by affinity chromatography on oligo(dT)–cellulose. As shown in Table II, no significant changes in the poly(A)-RNA content were observed between mid-exponential phase and stage IV of sporulation. This result disagreed with the report by Kerjan & Szulmajster (1980) that poly(A)-RNA could be detected only in sporulating cells of *B. subtilis* and was absent from asporogenic strains. In order to ascertain whether the discrepancy between our results and those of Szulmajster may have been due to the use of different strains of *B. subtilis*, we repeated our experiments with the same strains as those used by Szulmajster [BGSC1A1 (sp$^+$ trpC2) and BGSC1S10 (spo OA12 trp C2)] and also with related strains. The results in Table III reveal no significant

Table III: Proportion of Pulse-Labeled Poly(A)-RNA during Exponential Growth and Stationary Phase of Different *B. subtilis* Strains[a]

strain[b]	description	% of poly(A)-RNA during exponential growth	% of poly(A)-RNA during stationary phase[c]
1A1	sp$^+$ trp C2	11	10
1A96	sp$^+$ trp C2 phe A1	14	13
1S10	spo OA12 trp C2	17	8
1S11	spo OA12 trp C2 tol B24	8	13
1S27	spo OJ87 met C3 tal-1	13	10

[a] Pulse labeling with [^{3}H]adenosine, cell lysis, and fractionation of RNA on oligo(dT)–cellulose were as described under Experimental Procedures. [b] Bacillus Genetic Stock Center designation. [c] 2 h after end of exponential growth.

differences in the levels of poly(A)-RNA in sporulating and exponentially growing cells of the sp$^+$ strains. Moreover, all asporogenic strains had significant amounts of poly(A)-RNA during both exponential and stationary phases.

DNA Hybridization Patterns of Poly(A)-RNA. An indication of the sequence complexity of pulse-labeled polyadenylated RNA could be obtained by hybridization to restriction endonuclease fragments of *B. subtilis* DNA, which had been separated according to size by agarose gel electrophoresis and immobilized on nitrocellulose filters (Southern, 1975). *B. subtilis* RNA was pulse labeled with [^{32}P]P_i either in mid-exponential cultures or 1 h after the onset of sporulation, and RNA was isolated by the proteinase K–sodium dodecyl sulfate procedure (Gopalakrishna et al., 1981). The RNA samples were separated into poly(A)-containing and unadenylated fractions by affinity chromatography on oligo(dT)–cellulose, and each fraction was subjected to a second affinity chromatography cycle to assure the elimination of possible cross contamination. Upon hybridization to immobilized *Eco*RI endonuclease fragments of *B. subtilis* DNA, the poly(A)-RNA fractions were found to anneal to many different DNA fragments, giving rise to complex banding patterns with significant differences between vegetative RNA and sporulating RNA (Figure 4A,C). Purified ^{32}P-labeled ribosomal RNA gave a strikingly different hybridization pattern (Figure 4E), whereas the addition of a large excess of unlabeled rRNA had no significant effect on the banding patterns produced by poly(A)-RNA (data not shown). Complex hybridization patterns were also obtained with the unadenylated RNA fractions (Figure 4B,D), with some bands coinciding with those seen with the corresponding poly(A)-RNA fractions. However, the degree of resolution achieved by unidimensional electrophoresis is not sufficient to permit conclusions concerning possible identity of individual bands.

Discussion

Affinity chromatography on oligo(dT)–cellulose of pulse-labeled RNA from *B. subtilis* has shown that 10–20% of rapidly labeled RNA contains polyadenylate sequences longer than 16 nucleotide residues, the minimum length for binding to the affinity matrix (Nudel et al., 1976). This value is somewhat higher than that reported by Graef-Dodds & Chambliss (1978) but less than that observed earlier in *B. brevis* (Sarkar et al., 1978). The presence of polyadenylate sequences was confirmed by the observation that about 14% of [^{3}H]adenosine-labeled RNA retained by oligo(dT)–cellulose at high ionic strength was resistant to hydrolysis by pancreatic and T_1 ribonucleases under conditions where [^{3}H]uridine-labeled RNA was completely hydrolyzed to acid-soluble ma-

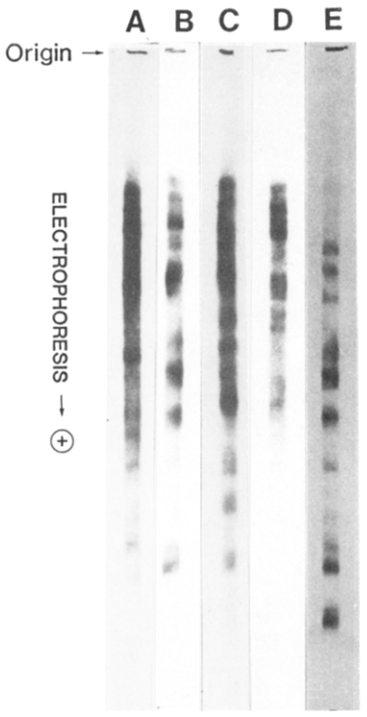

FIGURE 4: Hybridizaton of various RNA fractions with *Eco*RI fragments of *B. subtilis* DNA immobilized on nitrocellulose strips. ^{32}P-Labeled RNA was isolated from ^{32}P pulse labeled (1 mCi/mL, 5 min) vegetative (100 Klett units) or sporulating (t_1) culture and separated into poly(A)-RNA and nonpoly(A)-RNA fractions by two cycles of affinity chromatography on oligo(dT)–cellulose as described under Experimental Procedures. Nitrocellulose strips containing 6 μg of DNA were hybridized with (A) vegetative ^{32}P-labeled poly-(A)-RNA (100 000 cpm), (B) vegetative ^{32}P-labeled nonpoly(A)-RNA (60 000 cpm), (C) sporulating ^{32}P-labeled poly(A)-RNA (13 000 cpm), (D) sporulating ^{32}P-labeled nonpoly(A)-RNA (27 000 cpm), and (E) ^{32}P-labeled rRNA (45 000 cpm). With the exception of (E) all samples contained 500 μg of unlabeled rRNA during hybridization.

terial. Assuming that adenylate residues constitute one-fourth of the nucleotide residues of the remainder of the RNA molecule, we can calculate that the polyadenylate sequence, in order to account for 14% of the total adenylate residues, represents on the average 4% of the poly(A)-RNA molecule.

The weight-average chain length (49 nucleotide residues) of the poly(A) tracts in *B. subtilis* was about 20% lower than that found in *B. brevis* (Sarkar et al., 1978) but was similar to that found in *E. coli* (45 residues; Nakazato et al., 1975) and considerably longer than that in *Caulobacter crescentus* (13–17 residues; Ohta et al., 1978) and *Hyphomicrobium* (15–40 residues; Schultz et al., 1978). Kerjan & Szulmajster (1980) have reported that 75–80% of the poly(A) sequences from sporulating cells of *B. subtilis* are 160–180 nucleotides in length. However, their result is difficult to reconcile with the fact that the poly(A)-RNA, from which these sequences are presumably derived, sediments as a peak at 6 S and thus has an average chain length of only about 200 nucleotide residues. From our measurements of the average chain length (49 residues) and the ribonuclease-resistant portion of poly-(A)-RNA (about 4% of the total nucleotide residues), one can calculate an average size of pulse-labeled poly(A)-RNA of about 1200 nucleotide residues, a typical size for mRNA.

Analysis of the 3′ termini of the poly(A) tracts obtained by T_1 and pancreatic ribonuclease digestion of poly(A)-RNA pulse labeled with [^{3}H]adenosine suggested that 60–75% of these sequences had 3′-hydroxyl termini and thus were located directly at the 3′ ends of the poly(A)-RNA. This agrees with the results obtained with *B. brevis* (Sarkar et al., 1978) and implies that at least one pyrimidine or guanine nucleotide residue is interposed between the 3′ end and 25–40% of the polyadenylate sequences.

The measurement of poly(A) sequences in unlabeled *B. subtilis* RNA was made possible by the procedure of Bergmann & Brawerman (1980), which involves annealing with [^{3}H]poly(U) and selectively digesting unhybridized poly(U) with pancreatic ribonuclease. We found that total RNA isolated by the proteinase K method (Gopalakrishna et al., 1981) and by the phenol precedure of Chambliss & Legault-Demare (1977) contained 0.091% and 0.059% poly(A) sequences, respectively (Figure 2A), confirming our earlier conclusion that phenol extraction causes some preferential loss of poly(A)-RNA (Gopalakrishna et al., 1981). The poly(A) content of poly(A)-RNA purified by affinity chromatography on oligo(dT)–cellulose was found to be 3.9% (Figure 2B). This value was in excellent agreement with the value of 3.8% calculated from the ribonuclease-resistant portion of pulse-labeled poly(A)-RNA (Table I).[2] Making use of this value, we can compute the level of poly(A)-RNA in total *B. subtilis* RNA as 2.3%.[3] If we assume that mRNA constitutes 9% of total *B. subtilis* RNA (Salser et al., 1968), we can conclude that about 26% of *B. subtilis* mRNA is polyadenylated. This contrasts with the average value of 15% obtained for the poly(A)-RNA content of pulse-labeled RNA, a discrepancy that may be due in part to the fact that only 85% of the pulse-labeled RNA in *B. subtilis* is mRNA (Salser et al., 1968) and in part to incomplete recovery of poly(A)-RNA upon oligo(dT)–cellulose chromatography.

Graef-Dodds & Chambliss (1978) had attempted to assess the mRNA activity of poly(A)-RNA from *B. subtilis* by measuring the stimulation of amino acid incorporation into protein in a cell-free protein synthesizing system derived from *B. subtilis*. Poly(A)-RNA was found to enhance amino acid incorporation 3–4-fold over the endogenous level, but since the effect showed no clear dependence on poly(A)-RNA concentration, it was not possible to compare the efficacies of poly-(A)-containing and unadenylated RNA. We found the cell-free translation system from *E. coli* MZ9, developed by Arnaud et al. (1980), more suitable for our purpose, since it had very low endogenous translational activity and showed a good response to added mRNA, which was proportional to RNA concentration. Poly(A)-RNA was considerably more effective than total RNA in stimulating protein synthesis, an indication that poly(A)-RNA was enriched in mRNA. Indeed, the unadenylated RNA fraction showed very little mRNA activity, although the possibility that this low activity was due to inhibitory material not adsorbed to oligo(dT)–cellulose has not been eliminated. The ability of poly(A)-RNA to program protein synthesis, together with its rapid turnover rate and its lower proportion in total RNA than in pulse-labeled RNA, constitutes strong evidence that it represents an mRNA fraction.

The question whether poly(A)-RNA consists of a restricted subpopulation of mRNA molecules or whether it represents many mRNA species was addressed by examining its sequence complexity through hybridization to restriction endonuclease fragments of *B. subtilis* DNA according to the procedure of Southern (1975). Our results showed that poly(A)-RNA

[2] According to Table I, 13.5% of the radioactivity of [^{3}H]adenosine-labeled poly(A)-RNA was ribonuclease resistant, i.e., contained in the poly(A) tract. Assuming that the nonpoly(A) portion of poly(A)-RNA contains 25% adenosine residues, it follows that the poly(A) tract accounts for [13.5/[(100 − 13.5) × 4 + 13.5]] × 100 or 3.8% of the poly(A)-RNA molecule.

[3] This value is obtained by dividing the poly(A) content of total *B. subtilis* RNA (0.091%) by the poly(A) content of poly(A)-RNA (3.9%).

annealed to many different DNA fragments and accordingly had a high sequence complexity. The possibility that the hybridization pattern observed with poly(A)-RNA might have been due to contaminating unadenylated RNA was eliminated by subjecting the material to a second cycle of affinity chromatography on oligo(dT)–cellulose, a procedure that had no effect on the hybridization pattern. Moreover, contamination with ribosomal RNA was ruled out by the observation that the banding patterns obtained differed significantly from that seen with rRNA and were unaffected by the presence of a large excess of rRNA in the hybridization mixtures. On the other hand, the question whether a single polynucleotide sequence might be represented both in poly(A)-RNA and in unadenylated RNA is more difficult to answer. Although comparison of the hybridization patterns suggested the common presence of certain major bands in both types of RNA, the resolution was not sufficient to judge their identity. A definitive answer would require the use of a single, defined DNA sequence as a hybridization probe. Our recent discovery that poly(A)-RNA from *B. subtilis* can serve as template for the synthesis of complementary DNA by reverse transcriptase (Gopalakrishna & Sarkar, 1982) may make this kind of experiment possible.

In view of the evidence that poly(A)-RNA of *B. subtilis* has mRNA activity, it was interesting to determine whether poly(A)-RNA functioned specifically during either growth or sporulation. In agreement with the earlier observations of Graef-Dodds & Chambliss (1978) in *B. subtilis* and our own results with *B. brevis* (Gopalakrishna et al., 1981), we found no significant difference in poly(A)-RNA content in pulse-labeled RNA at various stages of growth and sporulation. This contrasts with the results of Kerjan & Szulmajster (1980), who were able to detect poly(A)-RNA only in sporulating cells of *B. subtilis* but not in exponentially growing cells nor in growing or stationary-phase cells of an asporogenic mutant. The discrepancy was not due to differences in bacterial strains, as we were able to detect significant amounts of poly(A)-RNA in exponentially growing cultures of the identical strains used by Kerjan & Szulmajster (1980). On the other hand, the results reported by these authors are difficult to interpret, not only because the RNAs from vegetative and sporulating cells were labeled for different time periods (3 and 30 min, respectively) and thus were not comparable entities but also because the isolation of RNA employed a phenol extraction protocol different from that used by Graef-Dodds & Chambliss (1978) or by us. It is well-known that considerable RNA degradation will occur in the course of phenol extraction procedures if adequate measures to inactivate ribonucleases are not taken; moreover, we have found that phenol extraction can lead to selective losses of poly(A)-RNA (Gopalakrishna et al., 1981).

Our observation and that of Graef-Dodds & Chambliss (1978), that poly(A)-RNA is found at all stages of growth and sporulation, suggest that the function of poly(A) sequences is not confined to a specific stage during bacterial differentiation but is more generally related to mRNA metabolism. The same seems to be the case in *C. crescentus* where poly-(A)-RNA can be found at all stages of growth and development (Ohta et al., 1978). Further evidence for a general role of poly(A) sequences comes from comparing the Southern blots of poly(A)-RNA from vegetative and sporulating cells (Figure 4). The hybridization patterns of the two types of poly(A)-RNA showed significant differences, indicating that poly(A)-RNA contained vegetative as well as sporulation-specific mRNA species. Although as little is known about the function of RNA polyadenylation in bacteria as in eucaryotes, we hope that the characterization of poly(A)-RNA in a simple organism that is amenable to genetic analysis may contribute to the elucidation of its function.

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