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Detection of high levels of polyadenylate-containing RNA in bacteria by the use of a single-step RNA isolation procedure

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ABSTRACT

A new one-step procedure for the isolation of bacterial RNA, involving lysis by proteinase K in the presence of sodium dodecyl sulfate, is described. Pulse-labeled RNA isolated by this procedure from *Bacillus brevis*, *Bacillus subtilis*, and *Escherichia coli* B has been found to contain a substantial fraction (15-40%) of polyadenylated RNA as determined by adsorption to oligo (dT)-cellulose. This contrasts with RNA isolated by procedures involving phenol extraction, a process which appears to lead to the selective loss of polyadenylated RNA. The presence of polyadenylated RNA in *E. coli* was confirmed by an independent method which involved hybridization with [^{3}H]polyuridylic acid. Using the proteinase K method for RNA isolation, it was possible to demonstrate the *in vitro* synthesis of polyadenylated RNA by toluene-treated cells of *B. brevis*, *B. subtilis*, and *E. coli*.

INTRODUCTION

The presence of polyadenylate sequences at the 3' end of mRNA was first observed in eucaryotic cells (1-3) and is now recognized as a characteristic feature of most eucaryotic mRNAs (4). In contrast, polyadenylated RNA has generally not been observed in procaryotes. The few reports of the occurrence of such material in bacteria have indicated either very low or variable levels of poly(A)-RNA (5-10) or relatively short poly(A) sequences (11). This observed dichotomy in the phylogenetic distribution of polyadenylated RNA has been interpreted in terms of the function of poly(A) sequences (12), but the possibility that the dichotomy may be only apparent and result from the failure of the isolation procedures for poly(A) RNA as applied to procaryotes has not been seriously considered.

In an earlier report, we had described the occurrence of substantial amounts of polyadenylated RNA (30-40% of pulse-labeled RNA) in *Bacillus brevis* with an average chain length of 60 adenylate residues (13). This material had been isolated by a new procedure involving proteinase K which avoided possible nucleolytic degradation and phenol extraction steps. In this paper, we compare the new procedure to the conventional method for

poly(A)-RNA isolation. We show that our new method involving proteinase K, unlike the older procedure employing phenol extraction, reveals a substantial proportion of polyadenylated molecules among RNA synthesized both *in vivo* and *in vitro*, not only in *B. brevis* but also in *Bacillus subtilis* and *Escherichia coli*.

EXPERIMENTAL PROCEDURES

Materials. Oligo(dT)-cellulose (Types T2 and T3) and oligo(dC)-cellulose were from Collaborative Research, oligo(dT)-cellulose (Type 7) from P-L Biochemicals, proteinase K from Beckman, heparin from Organon, and [^{3}H]poly(U) (5.2 Ci/mmole), [5-^{3}H]uridine (25 Ci/mmole), [2,8-^{3}H]adenosine (16.8 Ci/mmole), [5-^{3}H]uridine 5'-triphosphate, [2,8-^{3}H]adenosine 5'-triphosphate, and [α-^{32}P] cytidine 5'-triphosphate from New England Nuclear.

Bacterial growth. Bacteria were cultivated at 37°C on a rotary shaker, and growth was followed with a Klett-Summerson colorimeter using a no. 42 filter. *Bacillus brevis* ATCC 8185 and *Bacillus subtilis* ATCC 6051 were grown in a rich medium (14). *Escherichia coli* B was grown either in the same rich medium or in M9-phosphate medium (15).

Labeling of RNA. Two-ml samples of exponentially growing cultures (100-120 Klett units) or sporulating cultures, harvested 2 hours after the end of exponential growth (300-325 Klett units) were treated with [5-^{3}H]uridine or [2,8-^{3}H]adenosine (2-20 µCi). After 30 sec, labeling was terminated by the addition of 25 mM NaN_3 and rapid cooling to 2°C in an ice salt mixture. The cells were collected by centrifugation, washed once with cold culture medium containing 25 mM NaN_3, and were suspended in a buffer (0.2 ml) containing 80 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. Cell lysis was effected in 0.45 ml by supplementing the mixture with sodium dodecyl sulfate (0.5%), proteinase K (0.23 mg/ml), EDTA (10 mM), 1,10-phenanthroline (2 mM), and heparin (0.2 mg/ml) and incubating for 20 min at 37°C. In the case of *B. subtilis*, preliminary cell wall disruption was necessary for complete lysis. This could be achieved either by incubating a cell suspension in 15 mM Tris·HCl, pH 8.0, 8 mM EDTA, and 0.45 M sucrose with lysozyme (2 mg/ml) for 40 min at 2°C, or by subjecting the lysis mixture, from which proteinase K had been omitted, to sonic disruption in a Heat Systems Model 431B Cup Horn Sonicator for 5 min at 0°C prior to proteolysis. The proteinase K lysate was brought to 0.5 M with NaCl and either used directly for binding to oligo(dT)-cellulose or frozen quickly and stored at -80°C for later use.

Labeled rRNA and tRNA were isolated from exponentially growing cultures

that had been incubated with [^{3}H]uridine for at least 3 generations. Ribosomes and 100,000 x g supernatant were obtained from cell lysates prepared by sonication and were used for the isolation of rRNA and tRNA, respectively, by means of phenol extraction as described below.

Phenol extraction of RNA. RNA was extracted with phenol-$CHCl_3$-isoamyl alcohol (50:50:1) as described by Aviv and Leder (16), the distilled phenol having been equilibrated with 0.05 M Tris·HCl, pH 9.0 (3). RNA was precipitated from the final protein-free aqueous phase by the addition of 2 volumes of ethanol, followed by 20 hours at -20°C.

Binding to oligo(dT)-cellulose. Oligo(dT)-cellulose (17 mg per ml of labeled culture) was washed twice with 1N NaOH and twice with 10 mM Tris·HCl, pH 7.5, containing 0.5 M NaCl in a sterile conical centrifuge tube. The proteinase K-treated RNA sample (0.1 - 3 x 10^6 dpm), diluted to 1 ml with 10 mM Tris·HCl, pH 7.5, 0.5 M NaCl, was mixed with the oligo(dT)-cellulose, and the suspension was shaken for 30 min at 25°C (for *B. brevis* RNA) or for 60 min at 4°C (for *B. subtilis* and *E. coli* RNA). Incubation at the lower temperature for one hour did not increase the binding of *B. brevis* RNA to oligo(dT)-cellulose but significantly enhanced the binding of *B. subtilis* and *E. coli* RNA, presumably on account of the presence of shorter poly(A)-sequences (17). The cellulose was collected by centrifugation and washed 4-6 times with 10 mM Tris·HCl, pH 7.5, — 0.5 M NaCl. Polyadenylated RNA was eluted by two successive 15-minute extractions with sterile water at 45°C. No further radioactivity was released when the residual cellulose was extracted with 50% dimethylformamide. This batchwise procedure gave highly reproducible yields of poly(A)-RNA which were comparable to those obtained with a column fractionation method. The amount of oligo(dT)-cellulose used was in excess of that required to bind all poly(A)-RNA in the samples, and no difference in the level of polyadenylated RNA was seen with oligo(dT)-cellulose Types T2, T3, and T7.

Hybridization of [^{3}H]polyuridylate. Unlabeled RNA was isolated from exponentially growing cells of *E. coli* B in a rich medium (14) by lysis with proteinase K and sodium dodecyl sulfate as described above and two precipitations with ethanol. The annealing reaction with [^{3}H]poly(U) was carried out as described by Bergmann and Brawerman (18). Various amounts of unlabeled RNA were incubated for 15 min at 25°C with 2 pmoles of [^{3}H]poly(U) (10 nCi) in a final volume of 50 µl containing 10 mM Tris·HCl, pH 7.6, 200 mM NaCl, 5 mM $MgCl_2$, and 0.2% sodium dodecyl sulfate. The samples were then diluted with an equal volume of 200 mM KCl and 5 mM $MgCl_2$, chilled to 4°C for 20 min, and centrifuged at 4°C to remove the precipitated potassium dodecyl sulfate. The super-

natant solution was then incubated for 30 min at 25°C with 2.5 µg/ml RNase A and 33 µg/ml DNase I (19). Carrier DNA (20 µg) was then added and the unhydrolyzed [^{3}H]polyuridylate was precipitated at 2°C with 2.5% trichloroacetic acid under conditions to minimize the lability of the tritiated polynucleotide (20). The precipitated material was collected on Whatman GF/C glass fiber filters and its radioactivity determined in a toluene-based scintillation fluid using a Beckman liquid scintillation spectrometer.

RNA synthesis in permeable cells. Permeable cells were prepared by treatment with 1% toluene for 10 min at 25°C as described earlier (21). RNA synthesis was carried out by incubating 6 x 10^8 cells per ml with 200 µM [^{3}H]ATP or 20 µM [^{3}H]UTP or [α-^{32}P]CTP, 2 mM each of the other ribonucleoside triphosphates, 100 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM 2-mercaptoethanol, and 0.4 mM potassium phosphate. After 15 min at 37°C, the reaction was terminated by the addition of sodium dodecyl sulfate and subjected to lysis by proteinase K as described above.

RESULTS AND DISCUSSION

Detection of poly(A)-RNA in bacterial lysates. When cells of *B. brevis*, *B. subtilis*, or *E. coli*, pulse-labeled with an RNA precursor, were subjected to lysis by proteinase K in the presence of sodium dodecyl sulfate, a substantial fraction of the radioactive RNA was adsorbed to oligo(dT)-cellulose at high ionic strength and eluted by water, indicative of the presence of polyadenylate sequences (Table 1). The fact that labeled RNA was bound to oligo(dT)-cellulose regardless of whether the radioactive precursor was [^{3}H]-adenosine or [^{3}H]uridine showed that the adsorbed material was polyadenylated RNA rather than free polyadenylic acid. The proportion of polyadenylated material among pulse-labeled RNA was highest in *B. brevis* (35-40%), both during exponential growth and sporulation, but substantial amounts were also found in *E. coli* B (25%) and in growing or sporulating *B. subtilis* (about 15%). When pulse-labeling was followed by a period of incubation with an excess of unlabeled precursor, the proportion of labeled polyadenylated RNA was reduced to less than 1 percent. This suggested that polyadenylated RNA turned over much more rapidly than bulk RNA, a property characteristic of mRNA.

The specificity of adsorption to oligo(dT)-cellulose for poly(A)-containing RNA was confirmed by the control experiments shown in Table 2. Pulse-labeled RNA bound much more extensively to oligo(dT)-cellulose than to oligo (dC)-cellulose, an indication that the interaction was primarily with the oligonucleotide moiety, presumably by base pairing, and not with the support-

TABLE 1: Proportion of poly(A)-RNA in pulse-labeled RNA from different bacterial species.

Organism	Stage of Growth	Labeled Precursor	% poly(A)-RNA[a]
B. brevis	Exponential	^{3}H-Ado	38
B. brevis	Exponential	^{3}H-Urd	30
B. brevis	Sporulating	^{3}H-Ado	37
B. brevis	Sporulating	^{3}H-Urd	26
B. subtilis	Exponential	^{3}H-Ado	16
B. subtilis	Exponential	^{3}H-Ado	0.2[b]
B. subtilis	Sporulating	^{3}H-Ado	12
E. coli B	Exponential	^{3}H-Ado	24
E. coli B	Exponential	^{3}H-Urd	18

[a] Percent of radioactive RNA adsorbed to oligo(dT)-cellulose at high ionic strength and eluted by water.

[b] A 1-hour incubation with unlabeled adenosine (0.4 mM) followed the pulse-labeling period.

ing matrix. Stable RNA species such as rRNA and tRNA, known not to contain poly(A) sequences, failed to bind to oligo(dT)-cellulose to a significant extent, even at 4°C. Further control experiments involving repeated binding

TABLE 2: Specificity of RNA binding to oligo(dT)-cellulose

RNA	Radioactive precursor	Substituted cellulose	Temp. of binding	% of RNA bound & eluted with H_2O
Pulse-labeled RNA *(B. brevis)*	^{3}H-Ado	oligo(dC)-cellulose	25°C	6
Pulse-labeled RNA *(B. brevis)*	^{3}H-Ado	oligo(dT)-cellulose	25°C	34
Pulse-labeled RNA *(B. brevis)*, unadsorbed to oligo(dC)-cellulose	^{3}H-Ado	oligo(dT)-cellulose	25°C	32
rRNA *(B. brevis)*	^{3}H-Urd	oligo(dT)-cellulose	25°C	0
rRNA *(B. brevis)*	^{3}H-Urd	oligo(dT)-cellulose	4°C	0
tRNA *(B. brevis)*	^{3}H-Urd	oligo(dT)-cellulose	25°C	0
rRNA *(E. coli)*	$^{32}P_i$	oligo(dT)-cellulose	25°C	0.1
rRNA *(E. coli)*	$^{32}P_i$	oligo(dT)-cellulose	4°C	1.3

to oligo(dT)-cellulose and measurement of ribonuclease resistance of poly(A)-RNA from *B. brevis* as well as the characterization of the poly(A) segment have already been published (13), and similar results have been obtained with poly(A)-RNA from *B. subtilis* and *E. coli* (unpublished experiments). Substitution of poly(U)-agarose for oligo(dT)-cellulose had no significant effect on the results.

These control experiments left little question that the material bound to oligo(dT)-cellulose at high ionic strength and eluted by water was indeed polyadenylated RNA. However, in order to be able to eliminate with confidence the possibility of artifact, we also determined the poly(A) content of bacterial RNA by a completely different procedure, which involved the hybridization of unlabeled RNA with [^{3}H]polyuridylic acid, followed by digestion with pancreatic ribonuclease (18). As shown in Fig. 1, the annealing of [^{3}H]poly(U) with total RNA extracted from *E. coli* B by the proteinase K procedure or with polyadenylic acid afforded protection from hydrolysis by RNase A in a manner dependent on the amount of RNA or polyadenylic acid used. Using polyadenylic acid as a standard, it could be calculated that 1 µg of total *E. coli* B RNA contained 0.14 ng of poly(A) sequences. The poly(A) content of poly(A)-RNA was estimated by treating [^{3}H]adenosine-labeled poly(A)-RNA from *E. coli*, isolated by adsorption to oligo(dT)-cellulose, with pancreatic and T_1 ribonucleases. It was found that 8% of the radioactive RNA remained acid-insoluble after pro-

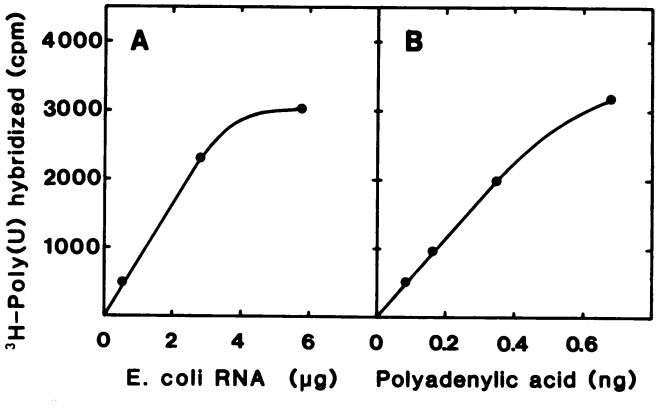

Figure 1: Measurement of polyadenylate sequences by hybridization with [^{3}H] polyuridylate. Various amounts of (A) total *E. coli* B RNA or (B) polyadenylic acid were annealed with [^{3}H]poly(U) and acid-insoluble radioactivity was measured after treatment with pancreatic RNase as described in "Experimental Procedures."

longed enzymic digestion, thus representing the poly(A)·stretches. Assuming that adenosine constitutes 25% of the nucleoside residues of the remainder of the RNA molecule, it could be calculated that the poly(A) sequences account for [8/(4 x 92 + 8)] or 0.021 of the poly(A)-RNA. Accordingly, 0.14 ng of poly(A) sequences correspond to 6.7 ng of poly(A)-RNA or 0.67% of the total cellular RNA. Since mRNA represents 1.3% of total *E. coli* B RNA (22), we can conclude that about 50% of *E. coli* B mRNA is polyadenylated. This is in good agreement with the observation that 18-24% of pulse-labeled RNA from *E. coli* B was bound to oligo(dT)-cellulose (Table 1), if one takes into consideration the fact that mRNA constitutes 40 to 50% of pulse-labeled RNA (23).

Further independent evidence for the presence of polyadenylated RNA in bacteria comes from the observation that *B. subtilis* RNA can act as a template for the oligothymidylate-dependent synthesis of DNA by reverse transcriptase from avian myeloblastoma virus (N. Sarkar and Y. Gopalakrishna, manuscript in preparation).

It should be noted that the content of polyadenylated RNA reported in Table 1 probably represents minimum values, and that the differences observed in various bacterial species may reflect differences in the recovery of poly(A)-RNA. Recovery would be affected by the level of nucleases in the different bacterial extracts and by the length of the poly(A)-stretch, which will affect the efficiency of binding to oligo(dT)-cellulose. Indeed, the average length of the poly(A) sequences in *B. subtilis* RNA is about 60% of that in *B. brevis*, and, as a result, the yield of the shorter poly(A)-RNA from *B. subtilis* was significantly enhanced by adsorption to oligo(dT)-cellulose at 4°C rather than at room temperature (results not shown).

Effect of extraction procedure on recovery of poly(A)-RNA. The high level of poly(A)-RNA found in bacterial pulse-labeled RNA contrasts with the much lower and variable levels seen by other workers. These have ranged from less than 1% of pulse-labeled RNA in *E. coli* (5) to 15% when the same organism was grown with limiting phosphate (6). In *B. subtilis*, one group of workers (8) reported a poly(A)-RNA content of 5-11% during growth and 3-6% during sporulation, while others (9) were unable to detect poly(A)-RNA in growing *B. subtilis* but found up to 20% during sporulation. A possible explanation for the consistently high levels of polyadenylated RNA observed by us is that our procedure, being very rapid and involving the use of ribonuclease inhibitors heparin and o-phenanthroline (18) as well as proteinase K, which will rapidly degrade other enzymes in the presence of sodium dodecyl sulfate, minimizes RNA degradation (24, 25). Indeed, in parallel experiments in which RNA was isolated by

the conventional procedure involving deproteinization by phenol extraction at pH 9 (3) rather than by proteinase K treatment, a poly(A)-RNA content of only 0.3% and 1% was found in pulse-labeled RNA from *E. coli* B and *B. subtilis*, respectively, even though the total recovery of pulse-labeled RNA was about 50%.

Several factors appear to contribute to the higher recovery of poly(A)-RNA by our procedure. One is the immediate exposure of pulse-labeled cells to proteinase K. If the cells were first frozen overnight at -80°C and then thawed prior to proteinase K treatment, the recovery of total labeled RNA was reduced by 40% and that of poly(A)-RNA by 55%. Moreover, phenol extraction itself seems to cause specific losses of poly(A)-RNA. This is shown by the results in Table 3 which compare the proportion of polyadenylated RNA before and after subjecting RNA isolated by the proteinase K procedure to phenol extraction. Even though more than 50% of total RNA was recovered after phenol extraction, 80% of poly(A)-RNA was lost.

It is not clear why the procedures that have been applied to the isolation of eucaryotic polyadenylated RNA cannot be successfully applied to bacteria. Undoubtedly, the relatively high level of nucleases, especially in *Bacillus* species, is an important factor. On the other hand, the selective loss of poly(A)-RNA during phenol extraction is difficult to understand. It may perhaps be due to differences in the structure of poly(A)-RNA in procaryotes and eucaryotes; indeed, the average length of the polyadenylate sequences is only about 60 nucleotide residues in *B. brevis* (13) and 35 residues in *B. subtilis* (unpublished observations) compared to an average of 200 nucleotides in mammalian mRNA (4). Notwithstanding these differences, our results show that a large fraction of bacterial mRNA is polyadenylated and that polyadenylation of mRNA must thus have a function in both procaryotes and

TABLE 3: Effect of phenol extraction on recovery of poly(A)-RNA from pulse-labeled *B. brevis*

Stage of growth of *B. brevis*	% Poly(A)-RNA[a] after proteinase K lysis	% Poly(A)-RNA[a] after proteinase K lysis and subsequent phenol extraction	% Loss during phenol extraction of total RNA	% Loss during phenol extraction of poly(A)-RNA
Exponential	39	10	50	87
Sporulating	18	4	39	87

[a]Percent of radioactive RNA adsorbed to oligo(dT)-cellulose at high ionic strength and eluted by water.

eucaryotes. Whether this function is the same remains to be established.

In vitro synthesis of polyadenylated RNA. The availability of an effective method for the isolation of bacterial polyadenylated RNA has provided a means for the study of poly(A)-RNA synthesis *in vitro*. As a first step, we have examined the polyadenylation of RNA in cells that were selectively permeabilized to small molecules by treatment with toluene. This system has the advantage that its macromolecular composition, including the DNA template and all enzymes, resembles that of the intact cell, yet that the concentration of small molecules, such as substrates and regulatory effectors, can be manipulated. In the presence of the four ribonucleoside triphosphates, one of which was radioactively labeled, toluene-treated cells of *E. coli* B, *B. brevis*, and *B. subtilis* were able to incorporate significant amounts of the labeled precursor into RNA which sedimented between 4S and 23S in a manner typical of mRNA isolated after pulse-labeling of growing cells (data not shown). Upon analysis of the RNA produced by binding to oligo(dT)-cellulose, 10-30% of the newly synthesized material was found to be linked to polyadenylate sequences (Table 4). The observation that the polyadenylated RNA could be labeled by any of the ribonucleoside triphosphates excluded the possibility that the product was simply polyadenylic acid. The synthesis of polyadenylic acid in permeable cells of *E. coli*, presumably mediated by polynucleotide phosphorylase, had been earlier observed by Deutscher (26). However, the following lines of evidence suggest that polynucleotide phosphorylase has no role in the poly(A)-RNA synthesis described by us: (a) Poly(A)-RNA synthesis was not stimulated by spermidine or inhibited by dADP, activators and inhibitors, respectively, of polynucleotide phosphorylase; (b) poly(A)-RNA synthesis was inhibited by high ionic strength which has no effect on polynucleotide phosphorylase; and (c) poly(A)-RNA synthesis was also observed in *E. coli* PR-7, a strain that

TABLE 4: Proportion of poly(A)-RNA synthesized *in vitro* by toluene-treated bacterial cells.

Organism	Labeled Substrate	%Poly(A)-RNA[a]
B. brevis (exponential)	^{3}H-ATP	29
B. brevis (sporulating)	^{3}H-ATP	28
B. subtilis (exponential)	[α-^{32}P]-CTP	10
E. coli B (exponential)	^{3}H-UTP	15

[a]Percent of radioactive RNA adsorbed to oligo(dT)-cellulose at high ionic strength and eluted by water.

lacks polynucleotide phosphorylase and is unable to mediate polyadenylic acid synthesis (26, 27). Our results thus represent the first report of the *in vitro* synthesis of polyadenylated RNA in a bacterial system and open the way for the characterization of the enzymic steps involved in the polyadenylation of RNA.

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