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Presence of polyriboadenylate sequences in pulse-labeled RNA of *Escherichia coli*

[oligo(dT)-cellulose chromatography/post-transcriptional modification/Millipore binding capacity/high-salt lysing medium]

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ABSTRACT **Pulse-labeled RNA isolated from *E. coli* cells grown on limiting phosphate medium and phosphate-containing medium was analyzed by oligo(dT)-cellulose chromatography and by Millipore binding assay for polyriboadenylate-containing RNA. Whereas poly(A)-containing RNA amounted to as much as 15% of the total pulse-labeled RNA from cells grown on limiting phosphate medium, pulse-labeled RNA from cells grown on phosphate medium gave values around 1.5%. Steady-state labeled RNA from cells grown on limiting phosphate medium contained 1.2% poly(A) RNA. The addition of poly(A) sequences appears to be post-transcriptional. These results strongly favor the view that bacterial mRNAs may contain poly(A) stretches.**

Considerable evidence has recently accumulated which indicates that messenger RNA and heterogenous nuclear RNA of eukaryotic cells (1–7), as well as viral (8–12) and viral-directed RNAs (3, 13), contain polyriboadenylate sequences 50–200 nucleotides long covalently linked to the 3′-OH terminus. Addition of poly(A) appears to occur post-transcriptionally (14–16). However, the presence of poly(A) in bacterial mRNA appears to be controversial. While Edmonds and Kopp (17) have reported that steady-state labeled total RNA of *Escherichia coli* contains poly(A) levels to an extent of 0.05%, Perry *et al.* (18) failed to detect poly(A) sequences in mRNA obtained from polysomes of pulse-labeled *E. coli* cells. Apart from variation in the labeling period, a basic difference between these two investigations is the medium in which the cells were labeled. Edmonds and Kopp used a phosphate-deficient medium for labeling the cells with $^{32}PO_4^{2-}$, whereas the latter workers employed a regular salts medium containing phosphate and labeling occurred with [^{3}H]nucleosides. During the studies of a novel poly(A) polymerase from *E. coli* PR7 (RNase I$^-$, *pnp*) we noted (*a*) that the enzyme in the early stages of purification is always contaminated with a poly(A)-degrading enzyme which could be inhibited by high salt (19, 20), and (*b*) that the poly(A) polymerase catalyzes efficient phosphorolysis of poly(A) in the presence of high concentrations of phosphate (20). Hence, we considered that these two interfering activities must be controlled in any investigation on the nature of poly(A) stretches in bacterial cells. In this communication we provide evidence for the occurrence of poly(A) sequences in pulse-labeled RNA extracted in the presence of high salt from *E. coli* cells grown in low phosphate medium.

MATERIALS AND METHODS

Growth and Labeling of Cells. *E. coli* PR7 (kindly provided by Drs. Gilbert and Reiner), and *E. coli* B were grown either in a modified limiting phosphate medium (21), or in M9-phosphate medium (22), both supplemented with 0.5% bactopeptone (Difco), and 0.2% glucose. The generation time of the two strains in limiting phosphate medium was twice as long as that in M9 medium. Cells were grown with shaking at 30°, and when optical density at 600 nm reached 0.5, 10 ml of cells were labeled for 2 min with [^{3}H]adenosine (200 μCi, 21 Ci/mmol), or [^{3}H]uracil (200 μCi, 14 Ci/mmol) or carrier-free $^{32}PO_4$ (250 μCi). The cells were immediately mixed with an equal volume of a chilled solution containing chloramphenicol, sodium azide, and KCl (final concentrations: 100 μg/ml, 25 mM, and 0.5 M, respectively), and frozen in dry ice–ethanol. The cells were thawed, harvested by centrifugation at 12,000 × *g* at 4°, and washed twice with 0.05 M sodium acetate buffer, pH 5.5, containing 0.5 M NaCl.

Extraction of RNA. The labeled cells were mixed with about 0.5 g of unlabeled cells grown under the *same* conditions, ground with 1 g of alumina, suspended in 2–3 ml of 0.05 M sodium acetate buffer containing 0.5 M NaCl, and clarified by centrifugation.

The supernatant solution was mixed with 10% sodium dodecylsulfate to give a final concentration of 0.5%, treated with an equal volume of freshly distilled phenol, agitated vigorously for 30 sec, allowed to stand for 10 min, and agitated for 30 sec. The suspension was treated with 1 volume of $CHCl_3$, mixed vigorously for 30 sec, and allowed to stand for 5 min, and mixed for 30 sec. The aqueous layer obtained after centrifugation at 12,000 × *g* for 5 min was again deproteinized with $CHCl_3$, mixed with 2 volumes of 95% C_2H_5OH, and allowed to stand overnight at −20°. Precipitated RNA was collected by centrifugation, dissolved in 1 ml of 0.01 M Tris–Cl buffer (pH 7.5) containing 2 mM $MgCl_2$, treated with electrophoretically pure DNase (10 μg), incubated for 30 min at 4°, and the solution was subjected twice to deproteinization with $CHCl_3$. RNA in the aqueous layer was precipitated with C_2H_5OH, collected by centrifugation at 12,000 × *g*, dissolved in 1 ml of Tris–Cl buffer (pH 7.5) containing 0.5 M KCl, and dialyzed twice against 100 volumes of the same buffer for a period of 1 hr. The yield of RNA was 30–40% based on radioactivity, and had a $A_{260/280}$ ratio over 2.0.

Oligo(dT)-Cellulose Column Chromatography. The procedure described by Aviv and Leder (23) was used with some modifications. The RNA solution was loaded on an oligo(dT)-cellulose column (Type T2, Collaborative Research, Waltham, Mass., 9 × 0.8 cm) previously equilibrated with 0.01 M Tris buffer + 0.5 M KCl at a flow rate of 6 ml/hr and 0.5 ml fractions were collected. The column was washed successively with 15 ml of 0.01 M Tris buffer + 0.5 M KCl,

Abbreviation: poly(A), polyriboadenylate.

Table 1. Millipore binding capacity of RNA fractions from oligo(dT)-cellulose columns

Eluate	Acid-insoluble (cpm)	Bound to Millipore (cpm)	% Bound
Exp. 1: Labeled with [^{3}H]adenosine			
(*a*) 0.01 M Tris buffer + 0.5 M KCl	3000	20	<1
(*b*) 0.01 M Tris buffer	625	724	100
(*c*) H_2O	290	310	100
Exp. 2: Labeled with [^{3}H]uracil			
(*a*) 0.01 M Tris buffer + 0.5 M KCl	2100	10	<1
(*b*) 0.01 M Tris buffer	310	330	100
(*c*) H_2O	400	435	100

E. coli PR7 cells were labeled for 2 min with either [^{3}H]adenosine or [^{3}H]uracil and the isolated RNA was subjected to chromatography on oligo(dT)-cellulose columns. The three eluates from each experiment were pooled separately, RNA was precipitated, dissolved in Tris buffer + 0.5 M KCl and dialyzed against the same buffer. The total volume of the three RNAs was 1, 0.5, and 0.5 ml, respectively, and 10 μl samples were appropriately diluted for estimation of trichloroacetic acid insoluble radioactivity and Millipore binding capacity.

15 ml of 0.01 M Tris buffer, 15 ml of H_2O, and the absorbancy and radioactivity of each fraction was determined. Fractions from each washing were pooled, treated with 95% C_2H_5OH, and precipitated RNA was dissolved in 0.01 M Tris buffer and dialyzed against the same buffer.

Other Methods. Detection of poly(A) sequences by Millipore binding and the elution of RNA from Millipore filters was carried out according to Lee *et al.* (5). The presence of poly(A) in RNA was also detected by incubating with RNase A (10 μg/ml) and T1 RNase (50 units/ml) for 30 min at 37°, and assaying by either trichloroacetic acid precipitable radioactivity, or by Millipore binding assay. Radioactivity was measured in a Beckman scintillation counter with Omnifluor–toluene scintillation mixture.

RESULTS

Elution profiles of pulse-labeled RNA extracted from *E. coli* PR7 grown on limiting phosphate or M9-phosphate medium

Table 2. Rebinding of Millipore-selected poly(A)-containing RNA

	Acid-insoluble (cpm)	Bound to Millipore (cpm)	% Rebound
Exp. 1: Adenosine labeled			
Fraction (*b*)	730	850	100
Fraction (*c*)	400	410	100
Exp. 2: Uracil labeled			
Fraction (*b*)	420	500	100
Fraction (*c*)	680	720	100

The RNA eluates of Tris buffer and H_2O washes from the two experiments described in Table 1 were individually adsorbed to Millipore filters, and RNA was eluted with Tris buffer containing sodium dodecyl sulfate, precipitated, and dissolved in Tris buffer. After dialysis aliquots from each of the four samples were analyzed for trichloroacetic acid insoluble radioactivity and Millipore binding capacity.

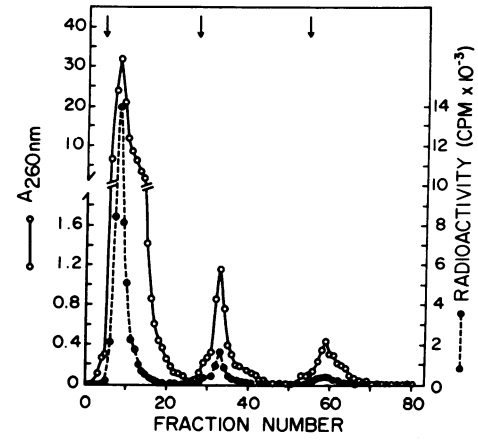

FIG. 1. Oligo(dT)-cellulose column profile. A culture (10 ml) of *E. coli* PR7 grown on limiting phosphate medium was labeled with [^{3}H]adenosine for 2 min, and RNA was extracted, and analyzed for poly(A) sequences. The arrows indicate the position of the buffer changes. For details, see *Materials and Methods.*

are shown in Figs. 1 and 2. On both media the bulk of the RNA was eluted with 0.01 M Tris buffer + 0.5 M KCl, but 0.01 M Tris buffer and H_2O eluates showed absorption at 260 nm or radioactivity only with RNA isolated from cells grown on limiting phosphate. Calibration of the column with radioactive samples of ribosomal RNA, tRNA, and authentic poly(A) showed that ribosomal and tRNAs are eluted in the high-salt wash, and that poly(A) can be eluted only with 0.01 M tris buffer. Similar results were observed with pulse-labeled RNA isolated from *E. coli* B cells. These experiments suggested the existence of poly(A) sequences in pulse-labeled RNA isolated from cells grown in low phosphate medium.

The three eluates from each experiment were pooled separately, and RNA was precipitated with C_2H_5OH, dialyzed, and assayed for acid insoluble radioactivity and Millipore binding capacity (Tables 1 and 2). Less than 1% of RNA from the high-salt wash was found to bind to Millipore filters, although this fraction contained over 85% of the total RNA. However, RNA isolated from Tris buffer and H_2O eluates bound to the Millipore filters almost completely. Thus these fractions contain RNA with poly(A) sequences. Similar

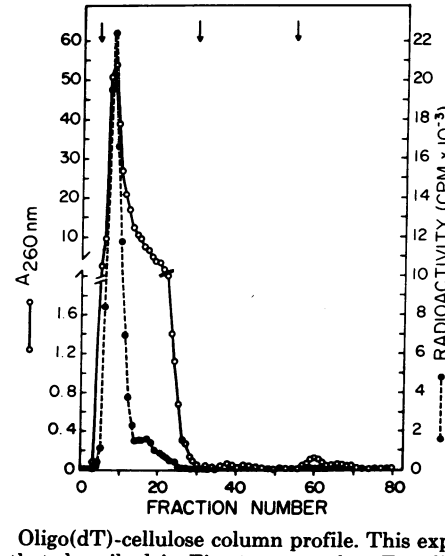

FIG. 2. Oligo(dT)-cellulose column profile. This experiment is similar to that described in Fig. 1 except that *E. coli* PR7 cells were grown in M9-phosphate medium.

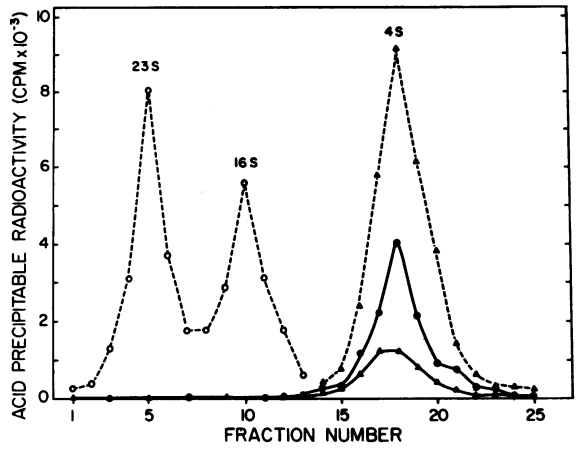

FIG. 3. Sedimentation analysis of RNase A and T1 RNase-resistant material. The RNA eluates of Tris buffer and H_2O washes (Table 1, Exp. 1) were treated with RNase A and T1 RNase for 30 min at 37°, and 10% sodium dodecyl sulfate was added to give a final concentration of 1%. The samples (0.1 ml) were layered on 5 ml of 5–20% sucrose gradients in 20 mM Tris–chloride buffer, pH 7.5, 0.1 M NaCl, and centrifuged for 5 hr at 45,000 rpm in SW 50.1 rotor. At the end of the run, 0.2 ml fractions were collected, precipitated with 5% trichloroacetic acid after addition of 50 μg of ribosomal RNA as carrier, filtered, and counted. RNase A and T1 RNase-resistant material from Tris buffer wash (●—●) and H_2O wash (▲—▲). Marker ribosomal RNA (○ - - - - ○) and tRNA (△ - - - - △).

results were obtained in cells labeled with adenosine or uracil. From the amount of radioactivity eluted in fractions (*b*) and (*c*), it could be calculated that about 14% of the RNA contains poly(A). Whereas most (94%) of the authentic poly(A) was eluted in the Tris buffer wash, the poly(A)-containing RNA was eluted in both Tris buffer and H_2O wash fractions. The reason for this difference is not clear. RNA bound to Millipore filters from fractions (*b*) and (*c*) was eluted, precipitated, dissolved in Tris buffer, dialyzed, and

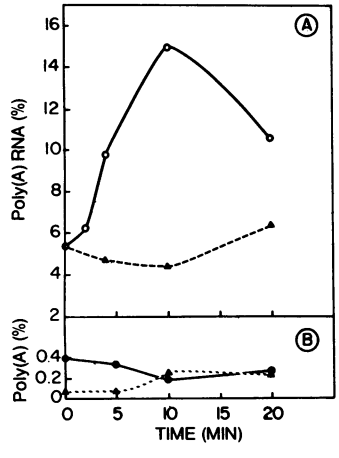

FIG. 4. (A) Time course of synthesis of poly(A)-containing RNA as analyzed by oligo(dT)-cellulose column chromatography. ○—○, after 2 min labeling with [^{3}H]uracil in limiting phosphate medium, rifampicin and unlabeled uracil were added. △ - - - - △, after identical labeling, rifampicin, uracil, and phosphate (70 mM) were added. (B) Time course of synthesis of poly(A) analyzed by RNase and T1 RNase treatment. ●—●, *E. coli* PR7 cells were labeled for 2 min with $^{32}PO_4^{2-}$ in limiting phosphate medium, and RNA was extracted by the lysozyme method in the absence of 0.5 M KCl. ▲ - - - - ▲, *E. coli* PR7 cells were labeled with [^{3}H]adenosine in M9-phosphate medium, and RNA was extracted by the lysozyme method in the presence of 0.5 M KCl.

Table 3. Millipore binding capacity of RNA fractions from *E. coli* grown on M9-phosphate medium

Fractions	Acid-insoluble (cpm)	Bound to Millipore (cpm)	% Bound
E. coli PR7			
(*a*) 0.01 M Tris buffer + 0.5 M KCl	266,000	940	<1.0
(*b*) 0.01 M Tris buffer	2,800	3,020	100
(*c*) H_2O	1,020	1,030	100
E. coli B			
(*a*) 0.01 M Tris buffer + 0.5 M KCl	201,000	1,150	<1.0
(*b*) 0.01 M Tris buffer	1,940	1,980	100
(*c*) H_2O	730	740	100

E. coli PR7 and *E. coli* B cells grown in M9-phosphate medium were labeled for 2 min with [^{3}H]adenosine and the isolated RNA was chromatographed on oligo(dT)-cellulose columns. The three eluates from each experiment were processed as described in *Materials and Methods* and then analyzed for trichloroacetic acid insoluble radioactivity and Millipore binding capacity.

assayed for acid-insoluble radioactivity and for Millipore binding. The results presented in Table 2 demonstrate that these RNAs bind with essentially 100% efficiency.

In other experiments with adenosine-labeled cells, the RNAs that were eluted with Tris buffer + 0.5 M KCl, Tris buffer, and H_2O washes were analyzed for their poly(A) content by digestion with RNase A and T1 RNase. Whereas only about 0.5% of the material that was eluted in high salt was resistant to this treatment, 30–50% of the labeled RNA which was eluted with Tris buffer and H_2O withstood enzymatic hydrolysis. Since this value appeared to be high, the RNase-resistant material was examined for possible contamination by DNA which might have survived the original DNase treatment. However, all the radioactivity was found to be acid-soluble after KOH hydrolysis (18 hr at 37° in 0.3 N KOH). A similar alkaline hydrolysate of RNase A and T1 RNase resistant material isolated from cells pulse-labeled with $^{32}PO_4^{2-}$ contained 89.3%, AMP; 1.9%, CMP; 5.9%; GMP; 2.9%; UMP.

The poly(A) segments, obtained by RNase A and T1 RNase treatment of Tris buffer and H_2O eluate fractions, were analyzed by velocity sedimentation in sucrose gradients and were found to cosediment with marker tRNA (Fig. 3). Although these results suggest that the poly(A) sequences may be as large as 4 S, the possibility that they may not be homogenous and may contain other residues interspersed therein must be considered and requires further study.

Pulse-labeled RNA from *E. coli* B and *E. coli* PR 7 cells grown on M9-phosphate medium was also chromatographed on oligo(dT)-cellulose columns (Table 3). Again, most of the labeled RNA was eluted with high salt. Tris buffer and H_2O eluates contain radioactive RNA which binds efficiently in the Millipore binding assay. However, the labeled RNA eluted in these fractions amounts to less than 1.5% of the total RNA. Thus, even in cells grown on high phosphate medium the presence of poly(A)-containing RNA was demonstrated, although the level appears to be low.

Further studies were directed towards obtaining evidence for a post-transcription addition of poly(A) to RNA. Exponentially growing cells in limiting phosphate medium were labeled with [^{3}H]uracil for 2 min, and further incorporation

of label and synthesis of RNA was arrested by the addition of unlabeled uracil (100 μg/ml) and rifampicin (200 μg/ml). At various intervals, samples were removed and cells were treated with lysozyme in high-salt buffer and lysed with sodium dodecyl sulfate, and RNA was extracted with hot phenol and $CHCl_3$. RNA samples were examined for poly(A)-containing RNA by oligo(dT)-cellulose column chromatography. The results are presented as percent poly(A)-containing RNA in Fig. 4, panel A, upper curve. The sample that was removed immediately after the addition of rifampicin (zero min) already contained 5% poly(A)-containing RNA. The level of poly(A) RNA steadily increased to 15% at 10 min and decreased to 10% at 20 min. The concentration of rifampicin used blocked RNA synthesis completely. Thus, the gradual increase of poly(A)-containing RNA is attributable to the addition of poly(A) sequences to RNAs that were labeled before the addition of rifampicin.

A parallel experiment was also carried out in which the pulse-labeled cells received rifampicin, unlabeled uracil, and phosphate. Cells were removed for RNA isolation at the specified intervals and analyzed by oligo(dT)-cellulose columns. The results are shown in Fig. 4, panel A. Whereas in the former experiment (limiting phosphate) the level of poly(A) RNA increased almost 3-fold at 10 min, poly(A)-containing RNA in the phosphate-containing medium did not significantly change from the level observed at the time of drug addition.

The procedure described above enabled us to investigate the importance of high salt in the initial extraction medium and to reexamine the time course of poly(A) addition to pulse-labeled RNA in cells grown on M9-phosphate media. The method was further simplified by analyzing the isolated RNA for RNase A and T1 RNase resistant trichloroacetic acid insoluble material.

The results presented in Fig. 4, panel B, reinforce the necessity of including salt in the lysing medium for extraction of RNA. In this experiment cells were pulse-labeled with [^{3}H]adenosine in limiting phosphate medium, samples were removed at the indicated time intervals after the addition of rifampicin, and lysed in buffer without KCl. The poly(A) content of the RNA varied between 0.3 and 0.4%. In the other experiment cells were grown in M9-phosphate medium, and pulse-labeled with $^{32}PO_4^{2-}$. Rifampicin was added, and samples were removed for extraction of RNA in high-salt lysing medium. The poly(A) content of these RNA samples was markedly diminished, although the level of poly(A) increased with time, and at 10 min RNase-resistant material was 0.23% compared to the zero time value of 0.06%. These results confirm the previous observations (Fig. 2 and in Table 3) of low levels of poly(A) in cells grown in M9-phosphate medium.

DISCUSSION

The results described above indicate the presence of poly(A)-containing RNA in *E. coli* cells grown on limiting phosphate medium as shown by pulse-labeling with either adenosine, uracil, or $^{32}PO_4^{2-}$. Whereas the presence of free poly(A) cannot be excluded by the adenosine and $^{32}PO_4^{2-}$ labeling experiments, the results obtained with [^{3}H]uracil are in accord with the interpretation that pulse-labeled RNA contain a population of RNA species with poly(A) sequences. Steady-state labeled RNA was also isolated from cells by labeling for 2.5–4 generations in limiting phosphate medium and analyzed by oligo(dT)-cellulose chromatography. Only 1.2% of the total radioactivity was eluted in Tris buffer and H_2O eluates. Recently, Nakazato *et al.* (24) have reported that poly(A)-containing RNA may amount to 0.2% of the total pulse-labeled RNA. We find higher values than this in steady-state labeled RNA, and as much as 15% poly(A)-containing RNA in pulse-labeled RNA.

The inclusion of high salt in the extraction buffers appears to be essential for the preservation of poly(A) sequences. As pointed out previously, poly(A) polymerase in the early steps of purification is always contaminated with a poly(A)-degrading enzyme (19, 20) which can be inhibited by raising the salt concentration. Furthermore, Rosenfeld *et al.* (25) have reported that rapidly labeled polyribosomal RNA isolated from lymphocytes or adrenal cortex is contaminated with a ribonuclease which cleaves the polyadenylate portion and retains activity despite exposure to sodium dodecyl sulfate and phenol. Whether such a nuclease also exists in *E. coli* remains to be established. The presence of a poly(A)-degrading nuclease active even at 0.5 M KCl has been observed by Milanino *et al.* (26) in their preparations of a purine polyribonucleotide synthetase from *E. coli*. Thus the actual levels of poly(A)-containing RNA may even be higher than the values obtained in our experiments.

Pulse-labeled RNA isolated from cells grown in phosphate-containing medium does contain poly(A) sequences although the level appears to be low. Addition of phosphate to pulse-labeled cells grown in low phosphate medium arrests further addition of poly(A) but does not decrease the original level. These preliminary experiments tend to suggest that in high phosphate medium either the poly(A) stretches are short, or they may be rapidly turned over. Thus, a careful study of poly(A)-containing RNA under a variety of physiological conditions is required before any definite conclusion can be drawn.

While we have shown that pulse-labeled RNA contains poly(A) stretches, and the addition may be post-transcriptional, we have yet to prove that specific messenger RNAs have poly(A) sequences. However, it is encouraging to point out that the 3′ terminus of a minor fraction of *in vivo* labeled T7 RNAs contains one or several adenylic acid residues (27).

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