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Poly(Adenylic Acid) Sequences in the RNA of *Caulobacter crescentus*

(RNA synthesis/dT-cellulose/gel electrophoresis/bacterial development)

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ABSTRACT **Poly(adenylic acid) sequences have been isolated from the Gram-negative bacterium *Caulobacter crescentus*. Most of these A-rich tracts are associated with large RNA molecules that constitute an important fraction of the unstable RNA in these bacteria, and, as estimated by poly(U) filter binding, they are not present in the 16S or 23S ribosomal RNA. Preliminary estimates of size from polyacrylamide gel electrophoresis suggest that the majority of the A-rich tracts ranges from 15 to approximately 50 residues in length.**

Poly(A) tracts are ubiquitously associated with a large fraction of the heterogeneous nuclear RNA and messenger RNA in mammalian cells (see review, ref. 1), and they are also found in yeast (2), slime mold (3), and plant cells (4). Their presence in *Escherichia coli* has not been established (5), however, and poly(A) has generally been considered a characteristic of the eukaryotic cell. We now report the presence of poly(A) tracts in the RNA (6) of the Gram-negative bacterium *Caulobacter crescentus* (see reviews 7 and 8). These sequences are shown to compose a significant fraction of the rapidly labeled RNA of this prokaryotic organism.

MATERIALS AND METHODS

Cell Growth. C. crescentus, strain CB15 (ATCC 19089), was generally grown in M3 salts (7) supplemented with 2 g of glucose per liter. Cells to be labeled with $^{32}PO_4$ were grown in G1, an imidazole-buffered medium (9) with 0.5 mM phosphate, and later transferred to G1 medium that contained 0.05 or 0.1 mM phosphate for labeling. *E. coli* K12, strain D10, was grown in M63 medium as described previously (10).

Labeling. Cells were labeled with $^{32}PO_4$ (New England Nuclear Corp.), [^{3}H]deoxyguanosine (Schwarz/Mann, 5 Ci/mmol) and [^{3}H]adenosine (New England Nuclear Corp., 32 Ci/mmol), as described below. The radioactivity of samples was generally measured in a toluene-based scintillation fluid after precipitating on filters with 10% trichloroacetic acid and washing with ethanol.

Purification of RNA. Labeling of cultures was terminated by addition of an equal volume of cold buffer (10 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 10 mM sodium azide), and the cells were collected by centrifugation and resuspended in the same buffer. Following the addition of deoxyribonuclease (25 μg/ml) and lysozyme (800 μg/ml) the cells were broken by freezing and thawing. The solution was adjusted to 2% tri-isopropylnapthalene-sulfonic acid (Eastman Kodak Co.) and to 0.1 M Tris.HCl, pH 9, and then extracted with an equal volume of buffer-saturated phenol at 4°. The phenol phase was reextracted with the 0.1 M Tris buffer, the two aqueous phases were combined and adjusted to 0.1 M sodium acetate, and the RNA was precipitated with 3 volumes of ethanol. The resuspended RNA was treated for 60 min at 4° with 25 μg/ml of deoxyribonuclease and extracted two additional times with buffer-saturated phenol.

Isolation and Estimation of Poly(A)-RNA. The amount of poly(A)-RNA in purified RNA preparations was estimated either by the method of Sheldon *et al.* (11) or by a modification of this method in which the RNA is applied directly to poly(U) filters in a small volume of binding buffer (12). Poly(A) and poly(A)-RNA were isolated on oligo(dT)-cellulose columns as prepared and described by Kates (12).

Enzyme Digestion. Enzyme digestion with pancreatic ribonuclease A (Sigma), ribonuclease T1 (Worthington Biochemicals), and ribonuclease T2 (Sigma) was carried out as described (12) on RNA samples that had been heated for 10 min at 80° and chilled rapidly.

RESULTS

Detection of Poly(A) in C. crescentus. Poly(A)-containing RNA was readily detected in RNA prepared from whole cells by means of the poly(U) binding method (*Materials and Methods*). An exponentially growing culture of *C. crescentus* was labeled for either 30 sec or 30 min with [^{3}H]adenosine and the RNA was purified by phenol extraction and then assayed for binding to poly(U) filters (Table 1). At least 90% of the labeled material retained by the filters could be eluted with water, and essentially all of the radioactive materials applied to the filters was labile in 0.5 N KOH at 37°. When RNA from 30 sec pulse-labeled *E. coli* cells was examined under comparable conditions, the levels of poly(U) binding were very low compared to those for *C. crescentus* (Table 1). No binding could be detected in experiments when the cells were labeled under effective chase conditions (30 min label, see Table 1). Perry *et al.* (5) have attributed low levels of poly(U) binding from *E. coli* RNA to nonspecific adsorption to filters. The possibility must be left open, however, that these bacteria contain unstable poly(A) molecules, or that they have some RNA with A sequences too short to be detected by these methods.

Kinetics of Synthesis. Table 1 shows that the fraction of RNA that bound to poly(U) filters was significantly higher when the cells were labeled for 30 sec than when they were labeled for 30 min. This suggested that the poly(U)-binding

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TABLE 1. *Binding of [^{3}H]adenosine-labeled RNA to poly(U) filters*

RNA preparation	RNA applied (cpm)	RNA bound* High salt cpm	High salt %	After water elution cpm
C. crescentus†				
30 sec label	86,000	6500	7.6	43
C. crescentus				
30 min label	510,000	2200	0.43	204
E. coli				
30 sec label	720,000	498	0.07	—
E. coli‡				
30 min label	600,000	3	0	—
[^{14}C]Poly(A)	2,380	1800	76	18

* RNA samples were assayed for binding to two sets of duplicate poly(U) filters in 0.12 M NaCl. One set was prepared according to the standard poly(U)-binding procedure (12), and the second set was eluted with 5 ml of distilled water at 57° before measuring radioactivity.

† After treatment of RNA from this preparation (1.7×10^4 cpm) with 0.5 N KOH for 30 min at 30° no radioactive label was recovered as acid-precipitable material.

‡ [^{3}H]Adenosine is exhausted after 5–10 min of labeling under these conditions (20 μCi/ml without added carrier).

RNA is less stable than the bulk of RNA in *C. crescentus*. We confirmed this conclusion by following the accumulation of [^{3}H]adenosine into total RNA and poly(A)-RNA of exponentially growing cells (Fig. 1). Although the estimates of poly(A) content are probably somewhat low in this experiment (see legend, Fig. 1), the 7-fold decrease in the relative amounts of poly(A)-RNA during the 30 min of labeling is significant. A similar pattern of incorporation was observed when [^{3}H]-deoxyguanosine (Fig. 2B) and [^{3}H]uridine (not shown) were used as precursors. The binding of G- and U-labeled RNA to poly(U) filters also indicates that the poly(A) tracts are part of larger RNA molecules, a conclusion that is supported by other experiments presented below.

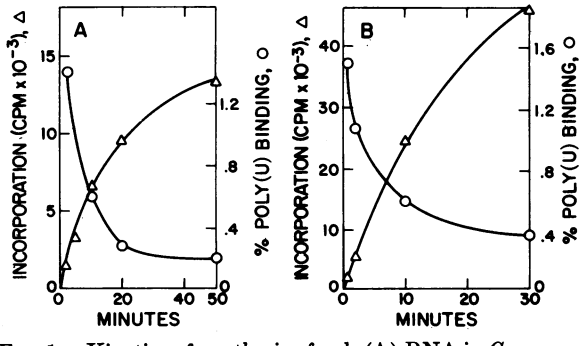

FIG. 1. Kinetics of synthesis of poly(A)-RNA in *C. crescentus*. (A) A 25 ml culture of exponentially growing cells was labeled with [^{3}H]adenosine (20 μCi/ml) in the presence of unlabeled adenosine (0.4 μg/ml). Aliquots of 5 ml were taken at the times indicated and used for the purification of RNA, as described in *Materials and Methods*. The RNA from each aliquot was suspended in 2 ml of buffer. Total incorporation was determined by analyzing 5 μl samples (△). Poly(U) binding was determined on duplicate samples of 0.2 ml and expressed as percentage of total incorporation (○). Estimates of poly(A) given in Figs. 1 and 2 were made according to the method of Sheldon *et al.* (11). These estimates were found to be about 50% lower on the average than values determined by a modification of this procedure (12) which was used in other experiments reported in this paper. (B) The experiment was carried out as described in "A" except that cells were labeled with [^{3}H]deoxyguanosine (20 μCi/ml).

Sedimentation Analysis of Poly(A)-RNA. The relative size of the poly(A)-RNA sequences was examined by sucrose gradient sedimentation analysis using RNA purified from *C. crescentus* cells that had been labeled for either 3 min or 30 min with [^{3}H]adenosine. Fractions from the gradients were pooled as shown in Fig. 2, and the RNA was collected by ethanol precipitation and then assayed by binding to poly(U). The bulk of the poly(A)-RNA sedimented between 4 and 5 S and 16 S. Most of the rapidly labeled RNA (presumptive messenger RNA) also sediments in this region of the gradient (3 min pulse, Fig. 2A), but at a somewhat higher average S value. The pattern of poly(U) binding strongly suggests that a substantial part of the unstable RNA and all or most of the 16S and 23S ribosomal RNA either lacks A tracts or contains tracts too short to allow detection by the binding assay.

Isolation and Characterization of Poly(A)-RNA. For isolation and characterization of poly(A)-RNA we prepared doubly labeled RNA from cells that had been grown for 30 min or 45 min in G1 medium with [^{3}H]deoxyguanosine and $^{32}PO_4$. The purified RNA was applied in 0.01 M Tris·HCl–0.12 M sodium chloride–1 mM EDTA, pH 7.5, to a oligo-(dT)-cellulose column to bind RNA with poly(A) sequences; the column was washed with the same buffer and then eluted with 0.01 M Tris–1 mM EDTA to release the poly(A)-RNA. The poly(A)-RNA isolated from different preparations of RNA accounted for 0.8–1.5% of the ^{32}P and 0.5–1.1% of the ^{3}H originally applied to the column. These figures agree with estimates made subsequently on the same RNA preparations by poly(U) filter binding, but they are somewhat higher than the values for poly(A)-RNA content shown above in Table 1 for [^{3}H]adenosine-labeled RNA from cells grown in M3 medium. Of the ^{3}H-^{32}P-double-labeled RNA isolated from the oligo(dT) column, approximately 60% was rebindable to poly(U) filters.

Poly(A) tracts were prepared from the purified poly(A)-RNA by digestion with ribonucleases A + T1. The percentage of A+T1-resistant radioactive material varied, depending upon the RNA preparation, but all of the labeled RNA was labile to base or to prolonged incubation with the addition of ribonuclease T2. The poly(A) from enzyme-digested samples was further purified by phenol extraction and rechromatography on a oligo(dT)-cellulose column as described above. Approximately one-half of the labeled material bound to the column and could be eluted in 0.01 M Tris, pH 7.5, buffer; this RNA was collected and characterized as described below. The RNA that failed to bind to the column at this step exhibited a ^{32}P/^{3}H ratio similar to the isolated bulk RNA and it was not examined further.

Comparison of Poly(A)-RNA and Poly(A) on Gel Electrophoresis. The size distributions of the poly(A)-RNA and the reisolated poly(A) tracts were compared by electrophoresis on 10% polyacrylamide gels. The poly(A)-RNA ran as a heterodisperse material between the 4S region and the top of the gel (Fig. 3). Since molecules larger than about 120,000 daltons would be excluded from the gel, 70–80% of the isolated poly-

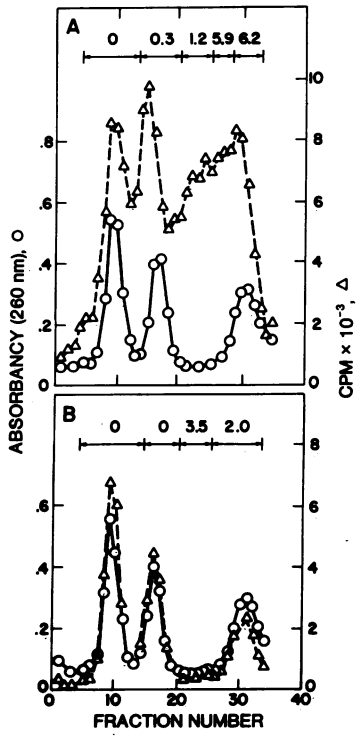

FIG. 2. Sedimentation analysis of poly(A)-containing RNA isolated from *C. crescentus* cells. A 10 ml culture of cells (3×10^8/ml) was labeled for (A) 3 min and (B) 60 min with [^{3}H]-adenosine (20 μCi/ml). Each culture was mixed with twice the number of unlabeled cells and RNA was purified by phenol extraction (*Materials and Methods*). The RNA was sedimented for 9 hr at 39,000 rpm on 5–30% sucrose gradients in an SW 39 rotor. Sedimentation is from right to left in the figures. Fractions from the sucrose gradients were analyzed for radioactivity (Δ) and pooled as indicated by the double-headed arrows. The pooled fractions were assayed for poly(A)-containing RNA by the filter binding method (11). Numbers above the arrows indicate the percentage of radioactivity in these pools which bound to poly(U) filters.

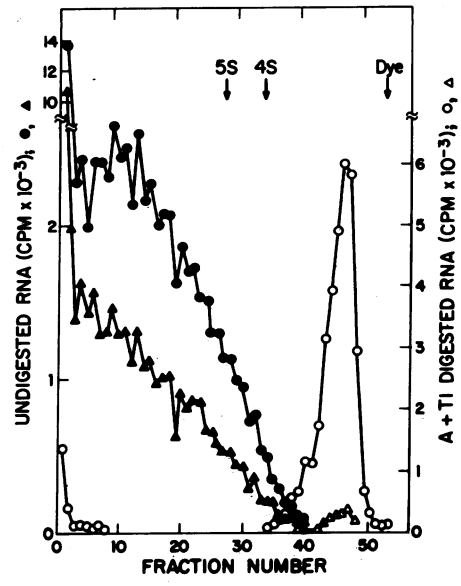

FIG. 3. Size distribution of poly(A)-RNA before and after ribonucleases A + T1 digestion. RNA was purified by phenol extraction from exponentially growing cells that had been labeled for 45 min with 0.1 mCi/ml of carrier-free $^{32}PO_4$ (O,●) and with 30 μCi/ml of [^{3}H]deoxyguanosine (Δ,▲). Poly(A)-RNA was isolated by chromatography on oligo(dT)-cellulose. The portion of the material that bound to oligo(dT) and eluted with 0.01 M Tris–1 mM EDTA, pH 7.5, was digested with nucleases A + T1 in 0.3 M NaCl for 30 min at 37°, phenol extracted, and repurified by binding to oligo(dT)-cellulose. The digested and undigested poly(A)-RNA were compared by electrophoresis on 10% acrylamide gels (12). The dye marker was bromphenol blue. The positions of 4S and 5S standards are indicated by arrows.

(A)-RNA would have a maximum length of 400 nucleotides, and the RNA not entering the gel would represent longer molecules. Thus, the bulk of the poly(A)-RNA, at least in these preparations, appears shorter than would be expected for the general population of messenger RNA molecules (see also Fig. 2). The poly(A) tracts examined under the same conditions migrated considerably faster than the poly(A)-RNA to give a single peak between the 4S RNA and the dye marker (Fig. 3). Electrophoresis on 14% polyacrylamide gels (Fig. 4) showed the poly(A) running as a broad band between the 4S peak and $(rA)_{10}$ and $(rA)_{15}$ standards. These findings are preliminary at this point, but they do suggest that the poly(A) tracts synthesized by *C. crescentus* during 45 min of labeling ($^1/_3$ generation) are significantly shorter than those found previously in eukaryotic cells. The size indicated here for both the poly(A)-RNA and the poly(A) tracts are minimum estimates for the molecules *in vivo*, however, since we have not eliminated the possibility of limited nuclease action during the isolation of the RNA from whole cells.

The sharp decrease in the $^3H/^{32}P$ ratio of the enzyme-treated RNA (Figs. 3 and 4) suggests that ribonucleases A + T1 have in fact preferentially digested the non-poly(A) sequences and that the "poly(A) tracts" do represent A-rich sequences. This conclusion is supported by initial results on the base composition of the ribonuclease-resistant RNA. Two-dimensional, thin layer chromatography (13) of an alkaline hydrolysate of one such ^{32}P-labeled RNA preparation (see

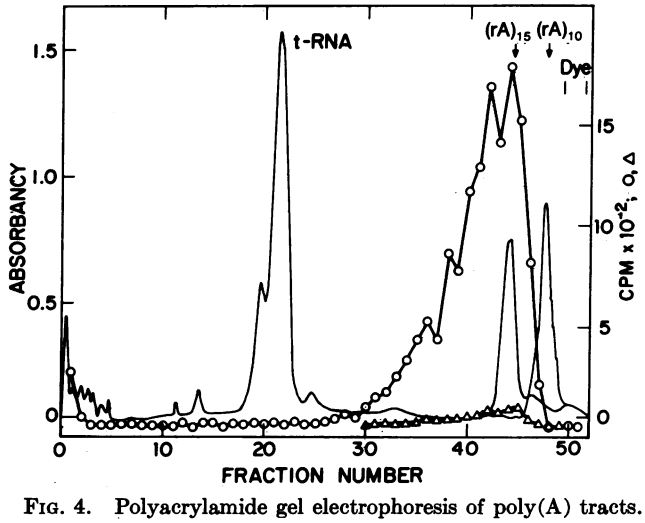

FIG. 4. Polyacrylamide gel electrophoresis of poly(A) tracts. Poly(A) tracts from RNA labeled with ^{32}P (O) and ^{3}H (Δ) were prepared as described in Fig. 3 and electrophoresed on 14% polyacrylamide gels. The positions of yeast transfer RNA and synthetic $(rA)_{10}$ and $(rA)_{15}$ (Collaborative Research) in parallel gels were determined by absorbance scanning.

Fig. 4) indicates that 2′:3′-adenylic acid accounts for 70–80% of the nucleotides present.

DISCUSSION

Our results show that *C. crescentus*, a Gram-negative bacterium, contains nucleotide sequences that are rich in adenylic acid, and that these sequences are associated with a metabolically unstable fraction of the cellular RNA. This unusual RNA reaches a relatively constant fraction of the labeled RNA during 30–45 min of incorporation, and as estimated by binding to either poly(U) filters or oligo(dT)-cellulose columns it constitutes approximately 1% of the ^{32}P-labeled RNA synthesized in this period. Several lines of evidence show that the "poly(A) tracts" are associated with other RNA sequences. First, the levels of binding to poly(U) filters and the kinetics of synthesis with this assay are similar whether [^{3}H]adenosine, [^{3}H]uridine, or [^{3}H]deoxyguanosine is the precursor (Fig. 1). Second, the poly(A) remaining after ribonucleases A + T1 treatment is drastically reduced in size compared to the intact poly(A)-RNA (Fig. 3). Finally, the sedimentation profile of poly(A)-RNA isolated from oligo-(dT)-cellulose columns is essentially unchanged as a result of either heat denaturation or treatment with dimethylsulfoxide (unpublished). The latter results indicate that the short A sequences are covalently bound to the larger RNA molecules.

Length estimates for the A-rich regions in *C. crescentus* RNA are tentative at this point. The mobility of the poly(A) on polyacrylamide gel suggests a size distribution ranging from approximately 15 to 50 nucleotides for the majority of molecules. The lower limit for tight binding to either poly(U) filters or oligo(dT)-cellulose is generally considered to be 20 nucleotides (5, 14). It might be expected, however, that A sequences of 13 to 20 residues would also bind, but much less stably than the longer A tracts (14).

The A sequences isolated from *C. crescentus* RNA are short compared to those found in eukaryotic cells. The length of the A tracts have been estimated as 200 in mammalian cells (1), 100 in *Dictyostelium discoideum* (15), and 50 in yeast (2). The tracts in all of these organisms are located at the 3′ terminus of the RNA. These sequences are added post-transcriptionally to the RNA, but an A tract of 25 nucleotides that is coded for by the genome has also been identified in *D. discoideum* (16). Another interesting observation in light of our findings with *C. crescentus* is the report of poly(A) tracts in the mitochondria of mammalian (17) and insect (18) cells. Many characteristics of these organelles, especially the biochemistry of the protein synthesizing apparatus, suggest that they may have evolved from a prokaryotic cell type, and, interestingly enough, the A tracts synthesized in mitochondria are much shorter than those attached to the messenger RNA of nuclear origin (17).

Since the presence of poly(A)-RNA has been previously established only in eukaryotic cells, the presence of what appear to be analogous sequences in a prokaryotic organism with rapidly turning over messenger RNA raises additional questions about the function of these A-rich tracts. We only know from the above results that the adenylate-rich sequences are found in an unstable fraction of the nonribosomal RNA of *C. crescentus* cells. To explore the function of the sequences we need to know more about their structure and about the metabolic role of the RNA with which they are associated. In connection with the second point, our special interest in *C. crescentus* is as a model system to study the regulation of development during the cell cycle (8). The hope is that these specialized RNA sequences will offer an approach to the study of the molecular aspects of this problem.

Note Added in Proof. Nakazato *et al.* (19) have now reported that 0.2% of the pulse-labeled RNA in *E. coli* is poly(A)-RNA.

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