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# Polyadenylated mRNA from the Photosynthetic Procaryote *Rhodospirillum rubrum*

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Total cellular RNA extracted from *Rhodospirillum rubrum* cultured in butyrate-containing medium under strict photosynthetic conditions to the stationary phase of growth has been fractionated on an oligodeoxythymidylic acid-cellulose column into polyadenylated [poly(A)$^+$] RNA and poly(A)$^-$ RNA fractions. The poly(A)$^+$ fraction was 9 to 10% of the total bulk RNA isolated. Analysis of the poly(A)$^+$ RNA on a denaturing urea-polyacrylamide gel revealed four sharp bands of RNA distributed in heterodisperse fashion between 16S and 9S. Similar fractionation of the poly(A)$^-$ RNA resulted in the separation of 23, 16, and 5S rRNAs and 4S tRNA. Poly(A)$^+$ fragments isolated after combined digestion with pancreatic A and $T_1$ RNases and analysis by denaturing gel electrophoresis demonstrated two major components of 80 and 100 residues. Alkaline hydrolysis of the nuclease-resistant, purified residues showed AMP-rich nucleotides. Through the use of snake venom phosphodiesterase, poly(A) tracts were placed at the 3′ end of poly(A)$^+$ RNA. Stimulation of [$^3$H]leucine incorporation into hot trichloroacetic acid-precipitable polypeptides in a cell-free system from wheat germ primed by the poly(A)$^+$ RNA mixture was found to be 220-fold higher than that for poly(A)$^-$ RNAs (on a unit mass basis), a finding which demonstrated that poly(A)$^+$ RNAs in *R. rubrum* are mRNAs. Gel electrophoretic analysis of the translation mixture revealed numerous $^3$H-labeled products including a major band ($M_r$, 52,000). The parent protein was precipitated by antibodies to ribulose bisphosphate carboxylase-oxygenase and comprised 6.5% of the total translation products.

The presence of adenylate-rich regions of various lengths at the 3′-OH end of the mRNA in *Eukaryota* has eased their isolation methodologically 1–3, 15, 35, 36, 41) and, in the process, the elucidation of their biological function with in vitro cell-free systems (28, 29, 43). This, in turn, has ushered in a virtual revolution in the studies of living systems at the molecular level. The importance of these residues is also reflected by the fact that they play a significant role in the preparation of DNA complementary to the mRNA with reverse transcriptase (5, 31, 42). In the past few years, a number of studies have shown that a population of RNAs in the non-photosynthetic procaryotes also contains these residues at the 3′-OH ends (9, 25, 27, 33, 38), and that such RNAs exhibit messenger properties when assayed under optimal conditions (11, 17).

In contrast to these reports, there is little available data on the occurrence and characteristics of analogous RNAs in the photosynthetic procaryotes (14). Currently, one of the major aims of this laboratory is to elucidate the biosynthesis and regulation of the enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (rubisco) of the photosynthetic and chemolithotropic procaryotes. Therefore the salient question of the involvement of polyadenylated [poly(A)$^+$] mRNA, which might be involved in coding for this enzyme, has been addressed. In *Rhodospirillum rubrum* grown in the light on butyrate-bicarbonate medium this enzyme comprises ca. 7% of the soluble protein (40), and maximal levels of rubisco are found after 5 to 6 days of growth under these conditions (34, 39). In this communication, we report the isolation, purification, and characterization of poly(A)$^+$ RNAs from 5-day cultures of *R. rubrum*.

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## MATERIALS AND METHODS

**Chemicals.** Common laboratory chemicals of reagent grade quality were procured from J. T. Baker Chemical Co. Biochemicals, enzymes, Tris, and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) were purchased from Sigma Chemical Co. Poly(A) markers were from Miles Laboratories, Inc. Ultrapure urea and gel electrophoresis grade acrylamide were products of Schwarz-Mann, Inc., and Bethesda Research Laboratories, Inc., respectively. [$^3$H]leucine and $Na_3{}^{32}PO_4$ came from New England Nuclear Corp., and [$^3$H]polyuridylate was from Amersham Corp. X-Omat AR films were from Eastman Kodak Co.

**Measurement of nucleic acid.** Nucleic acids were measured by dissolving the nucleic acid in 1 ml of sterile water or buffer in a cuvette with a 1-cm light path in a Beckman DU Spectrophotometer at 254 nm. A sample with an optical density at 254 nm of 20 was assumed to contain 1 mg of nucleic acid.

**Bacterial strains and growth conditions.** The *R. rubrum* strain and its culture for 5 days in liquid medium containing butyrate and biotin under strict photosynthetic conditions have been described previously (40). For labeling cell cultures, the medium having 0.1 of its normal phosphate concentration was inoculated first; 2 mCi of $^{32}PO_4{}^{3-}$ was then added to a 500-ml culture, adjusting the phosphate to its normal concentration of 0.64 M. Growth ensued, and cells were incubated for 120 h at 30°C.

**Extraction of total cellular RNA.** Several preliminary experiments indicated that preparation of undegraded polysomes after the cell rupture by traditional methods like grinding in a mortar with alumina or by sonic treatment is not satisfactory with *R. rubrum* because these procedures leave about one-third of the cells unbroken as judged by micro-

scopic examination. To circumbent this problem, total cellular RNA was extracted according to the following steps:

(i) The $^{32}$P-labeled or unlabeled cell pellet (2 g, wet weight) was washed and dispersed in the extraction buffer containing 100 mM Tris-chloride (pH 8.8, 25°C), 120 mM NaCl, 3 mM disodium EDTA and 2% sodium dodecyl sulfate (SDS). Sodium heparin (0.1 mg/ml) and diethylprocarbonate (0.01%, vol/vol) were added to reduce digestion of RNA by intracellular nuclease(s). The resulting viscous suspension was then subjected to freezing in a dry ice-methanol bath followed by thawing in cold water; this freezing-thawing was repeated for three more cycles. The total volume was made 0.5% with sodium deoxycholate, shaken gently, and incubated for 30 min at 30°C. Incubation was terminated by the addition of an equal volume of phenol-chloroform (1:1, vol/vol) and shaken vigorously for 90 min.

(ii) The mixture was clarified by centrifugation at 17,000 × *g* at 2°C for 15 min. The upper phase was carefully collected and saved in a clean, autoclaved tube at 4°C. The phenol-chloroform organic lower phase was mixed with 10 ml of extraction buffer and shaken for 15 min. The phases were separated by centrifugation as described above. The combined upper phases were extracted four times with the phenol-chloroform mix and shaken vigorously each time for 30 min. The final upper phase was collected and made 100 mM with 3 M potassium acetate (pH 6.5), and 2 volumes of 95% cold ethanol was added with gentle mixing (by tube inversion). The crude nucleic acid was allowed to precipitate at −20°C for 20 h.

(iii) The precipitate was collected by centrifugation at 10,000 × *g* (−5°C) for 10 min, dried by lyophilization, treated with 5 ml of 3 M potassium acetate (pH 6.5), and centrifuged at 17,000 × *g*. The supernatant was discarded. This washing procedure was repeated three times. The pelleted material was dissolved in 4.0 ml of 0.1 M potassium acetate (pH 5.5), precipitated with 2 volumes of cold 95% ethanol, and washed as described above. After three precipitations, the final pellet was washed with 95% chilled ethanol and left at −20°C for 90 min.

**Oligo(dT)-cellulose chromatographic fractionation of total nucleic acid.** Washed nucleic acid was collected by centrifugation, dried under nitrogen, and dissolved in 5 ml of binding buffer composed of 500 mM NaCl, 15 mM Tris-chloride (pH 7.5, 25°C), 0.3% lithium dodecyl sulfate, and 1 mM disodium EDTA. To disaggregate the RNA before chromatography, the solution was heated for 5 min at 50°C and brought to room temperature. It was then fractionated on 200 mg of oligodeoxythymidylic acid [oligo(dT)]-cellulose that had been presoaked, washed, and equilibrated with binding buffer and packed in a 1- by 10-cm Bio-Rad Econo siliconized column with a resin bed volume of 1 ml. The flow-through fraction was recycled thrice through the column at ambient temperature (22°C). Fractions (1 ml) were then collected, and the column was washed with binding buffer until all fractions absorbing at 254 nm had been eluted. When $^{32}$P-labeled nucleic acid was fractionated in an identical manner, a 2-μl sample was withdrawn from each fraction, and Cerenkov radiations were counted in a Beckman LS-9000 scintillation counter. Elution with binding buffer was continued until no more $^{32}$P was detectable. The material retained by the resin under these conditions was then eluted with diethylpyrocarbonate-treated, doubly-distilled sterile water at 22°C. Peak fractions that had been bound by the resin, as well as those from the flow-through, were separately combined, chilled, mixed with 2 volumes of 95% ethanol, and left to precipitate at −20°C for 15 h. Each precipitate was collected at 24,000 rpm with a Ti70.1 rotor in a Beckman L8-70 ultracentrifuge for 2 h, lyophilized, taken up in a minimal volume of sterile water, and frozen at −20°C.

**Electrophoretic analysis of putative poly(A)$^+$ and poly(A)$^-$ RNA species.** To examine the size distribution, the putative poly(A)$^+$ RNA, which had been retained by oligo(dT)-cellulose, and the flow-through fraction were subjected to 4 to 6% denaturing urea-polyacrylamide gel electrophoretic analysis, based in principle on the methodology of Maniatis et al. (22), with the following modifications. The equivalents of approximately 30,000 and 100,000 Cerenkov counts of poly(A)$^+$ and poly(A)$^-$ RNA, respectively, were each added to 25 μg of *Escherichia coli* tRNA, mixed, lyophilized, and kept frozen at −20°C. The denaturing gel was prepared with precalculated amounts of acrylamide and bisacrylamide (20:1, wt/wt) that had been dissolved with slight warming in an appropriate volume of 10-fold-concentrated TEB buffer (pH 8.2, 25°C), which contained 10.8 g of Tris, 0.93 g of disodium EDTA, and 5.5 g of boric acid. This solution was adjusted to contain 7 M urea, filtered, degassed, and polymerized by the addition of 0.5 ml of 10% freshly prepared ammonium persulfate and *N,N,N,N'*-tetramethylethylenediamine. The gel was cast as a 140- by 120- by 0.8-mm slab between two clean glass plates. After 3 h of polymerization, the gel was prerun with 0.1 strength TEB buffer for 2 h at 75 V. Before electrophoresis, the lyophilized samples to be analyzed were taken up in 40 μl of sample buffer consisting of 10 M urea, 0.1 strength TEB buffer, 15% sucrose, and a trace of bromphenol blue as dye marker, heated to 60°C for 4 min, rapidly cooled in ice water, and carefully applied onto the gel. The electrophoresis buffer was TEB. The gel was run at constant voltage (100 V) for 5 h at 22°C. After the run, one of the glass plates was removed, and the gel was covered with Saran Wrap, exposed to Kodak no-screen X-ray film, and placed at 4°C.

**Isolation and sizing of oligo(dT)-cellulose-retained poly(A) sequences.** The equivalent of approximately 185,000 Cerenkov counts of RNA retained by the resin was ethanol precipitated in the presence of 5 μg of carrier tRNA. The material was recovered, dried, and dissolved in 50 μl of 20 mM Tris-chloride, 20 mM NaCl, and 1 mM EDTA (pH 7.4, 25°C containing 20 μg of pancreatic RNase A per ml and 100 U of RNase $T_1$ per ml and drawn into a siliconized micropipette, which was then flame sealed at both ends. After incubation at 37°C for 35 min, reactions were terminated by chilling in ice, and the contents were blown into a microfuge tube and made 1% with SDS. The solution was extracted thrice with equal volumes of buffer-saturated phenol-chloroform (1:1, vol/vol). The combined upper phases, recovered after centrifugation in a Beckman microfuge B, were washed thrice with anhydrous ether and rechromatographed under conditions described above through an oligo(dT)-cellulose column at room temperature. The material retained by the resin was eluted with sterile water and precipitated by ethanol in the presence of 10 μg of carrier tRNA at −20°C overnight. The precipitate was recovered by centrifugation. The lyophilized material was dissolved in 10 μl of sterile water. A portion of 5 μl was analyzed on a denaturing urea-polyacrylamide slab gel as described along with poly(A)s of known size. At the end of the run, one face of the gel was carefully covered by Saran Wrap and exposed to Kodak X-Omat film at 4°C.

**Analysis of nucleotide composition of oligo(dT)-cellulose-bound and nuclease-resistant fragments.** A second portion of 5 μl of the oligo(dT)-cellulose bound fragments was made to 0.3 M NaOH and drawn into a siliconized capillary, flame

sealed at both ends, and subjected to hydrolysis at 37°C. After 18 h of incubation the hydrolysis was terminated by neutralizing the reaction mixture in a microfuge tube. The chromatographic separation of the total reaction products was performed on Whatman 3MM paper by high-voltage electrophoresis (32). The paper was dried in air and exposed to no-screen X-ray film.

**Digestion of poly(A)$^+$ RNA by snake venom phosphodiesterase and consequent changes in hybridizability with [$^3$H]polyuridylate.** The contents of two siliconized plastic tubes containing 1 μg each of the poly(A)$^+$ RNA fraction were dried by lyophilization, and each was dissolved in 49 μl of 5 mM Tris-chloride containing 10 mM $MgCl_2$ (pH 8.9, 25°C). After adjustment to 37°C, 1 μl of a solution containing 1 μg of snake venom phosphodiesterase was added to one tube (4), and 1 μl of water was added to the other tube as a control. At indicated times, the enzymic digestion was quenched by transfer of a 5-μl portion to 100 μl of cold 70% ethanol containing 0.1% diethylpyrocarbonate. The precipitate was washed free of salts with cold 70% ethanol, dried, and dissolved in 50 μl of hybridization buffer (10 mM Tris-hydrochloride [pH 7.5], 0.2 M NaCl, 5 mM $MgCl_2$) containing excess [$^3$H]polyuridylate (21, 41). After incubation at 35°C for 30 min, 10 μl was transferred to a Whatman GF-C filter paper disk, dried in air, and sequentially washed with 5 ml of 15, 7, and 5% cold trichloroacetic acid containing 1.3 μg of tRNA per ml. The processed disks were dried at 25°C, and the cold trichloroacetic acid-insoluble hybrids trapped on the filters were assayed with a Beckman LS-9000 liquid scintillation counter. In the untreated control, a 5-μl portion was processed exactly as described above for the counterpart reflecting enzyme digestion. The degree of hybridization detected in this sample was then the zero time control value.

**In vitro translation of poly(A)$^+$ and poly(A)$^-$ RNA fractions.** The poly(A)$^+$ and poly(A)$^-$ RNA fractions were assayed for in vitro coding by priming a cell-free, heterologous system that had been derived from untoasted wheat germ prepared by the method of Marcu and Dudock (24) and treated with micrococcal nuclease (150 U/ml) for 10 min at 20°C (29) to reduce endogenous mRNA activity. A typical translation reaction contained in 50 μl the following components in their final amounts: wheat germ extract, 20 μl; HEPES adjusted to pH 7.5 with KOH, 40 mM; potassium acetate, 112 mM; magnesium acetate, 2.5 mM; ATP, 1 mM; GTP, 25 μM; creatine phosphate, 8 mM; creatine phosphokinase, 5 μg; dithiothreitol, 1.5 mM; spermidine tetrahydrochloride (neutralized), 40 μM; 50 μM of each of 18 unlabeled amino acids except leucine; and 4 μCi of [4,5-$^3$H]leucine (1 μM) with a specific activity of 59.8 Ci/mmol. The individual components were assembled in 290- by 7-mm acid-washed, heat-treated, autoclaved glass tubes standing on ice and gently mixed by tapping after each addition. The reactions were primed with either 1.0 μg of poly(A)$^+$ RNA or 10 μg of poly(A)$^-$ RNA and incubated at 26°C. At appropriate times a 2-μl portion from each reaction mixture was pipetted into a chilled siliconized glass tube, and radioactive leucyl tRNA was digested in the samples by treatment with 5 μl of a mixture containing 100 μg each of pancreatic A and $T_1$ RNases per ml at 35°C for 30 min (15). The entire reaction mix was then spotted on Whatman 3MM paper disks, dried in air for 45 min, and immersed in 10% chilled trichloroacetic acid containing 5 mM cold leucine for an additional 45 min. The filter disks were then boiled for 15 min to liberate $^3$H-labeled leucyl tRNA fragments and cold leucine and cooled on crushed ice. After air drying, the disks were extensively washed sequentially in cold 80% acetone and 95 and 100% ethanol and finally with anhydrous ether followed by drying in air. The dried filter paper disks were assayed in 5 ml of xylene-based scintillation fluid in a Beckman LS-9000 counter.

**Immunoprecipitation of rubisco from in vitro translation products by specific *R. rubrum* rubisco antibody.** Immunoprecipitable rubisco after cell-free translation was detected in the following manner. Samples (20 μl) of the in vitro translation products were added to 180 μl of a buffer (pH 8.0) containing 10 mM sodium borate, 160 mM NaCl, 0.6% Triton X-100, 5 mM cold leucine, 40 μg of antibodies to homogeneous *R. rubrum* and rubisco prepared in rabbits, 10 μg of bovine serum albumin, and 25 μg of antiserum to rabbit immunoglobulin G. After incubation at 35°C for 1 h, the immunoprecipitate was further incubated at 4°C for 20 h. The precipitated material was collected by centrifugation for 10 min at 4°C, dissolved in 100 μl of the above buffer, and layered gently on discontinuous sucrose gradients prepared in washed microfuge tubes as described below. The sucrose solutions prepared in the borate buffer described above contained 100 μl of 1.0 M sucrose under 75 μl of 0.5 M sucrose. The gradient was centrifuged for 10 min at 7,700 × *g* at 4°C. The pelleted immunoprecipitate was collected and processed further for SDS-gel electrophoresis (19).

## RESULTS

**Fractionation and sizing of poly(A)$^+$ RNA.** Fractionation of the total RNA isolated by the procedure outlined above from butyrate-light-grown *R. rubrum* on an oligo(dT)-cellulose column resulted in a major unbound part and a relatively small proportion that was retained by the resin. From four trials, yields of the resin-bound fraction were 9.5 ± 0.5% of the purified, total RNA initially applied to the column whether measured by absorbance at 254 nm or after long-term labeling with $^{32}$P as described above. When RNA isolated as described above was heated in water to 73°C (instead of to 50°C in the presence of 0.5 M NaCl), and the solution was cooled and adjusted to 0.5 M in NaCl and then chromatographed in the presence of 0.5 M NaCl, the proportion bound was similar (Table 1). Upon chromatography of the eluate twice, small additional amounts of bound RNA were released as was the case when the oligo(dT)-cellulose column was washed with binding buffer (fourth elution) or with buffer containing a lower concentration of 0.12 M NaCl (fifth elution). These observations confirm those of Srinivasan et al. (38) made during the isolation of poly(A)$^+$ RNA from *E. coli*. Finally, elution with water released RNA that comprised 8.9% of that originally subjected to chromatography, and this fraction could be rechromatographed on fresh resin with almost no reduction in yield (Table 1).

Exploratory experiments showed that disruption of the cells by SDS without sequential freezing and thawing gave nucleic acid that bound to the oligo(dT)-cellulose matrix. However, the yield was invariably lower. Omission of heparin and diethyl pyrocarbonate in the extraction buffer used for cell disruption reduced the yield of total nucleic acid from this procaryote by 10-fold. The present recovery of oligo(dT)-cellulose-retained RNA of 9% after extraction by phenol-SDS is somewhat lower than values of 20.5 and 15% reported for *Bacillus subtilus* and *E. coli* (18, 38), respectively, but within the range of 1 to 20.5% isolated from various bacteria (12, 18, 21, 27, 38, 46) and spinach chloroplasts (43). The RNA fraction subjected to fractionation upon oligo(dT)-cellulose was free of high- and low-molecular-weight sheared

TABLE 1. Yield of poly(A)$^+$ RNA from *R. rubrum* grown on butyrate-bicarbonate for 120 h

| Chromatographic cycles on oligo(dT)-cellulose column[a] | % Remaining bound[b] |
|---|---|
| First elution | 11.2 |
| Second elution: recycling of unbound fraction from first elution | 10.0 |
| Third elution: recycling of unbound fraction from the second elution | 9.2 |
| Fourth elution with high salt: 15 mM Tris-chloride (pH 7.5) containing 0.5 M NaCl | 9.0 |
| Fifth elution with lower salt: 15 mM Tris-chloride (pH 7.5) containing 0.12 M NaCl | 8.9 |
| Sixth elution with sterile water | 0[c] |

[a] Before chromatography the sample was dissolved in 2.0 ml of water, heated to 73°C for 15 min, cooled to 23°C, and adjusted (in 5.0 ml) to 15 mM Tris-chloride (pH 7.5), 1 mM EDTA, 0.5 M NaCl, and 0.3% lithium dodecyl sulfate. A sample of 0.10 ml was removed and diluted with 9 volumes of binding buffer, and the optical density at 254 nm was measured. After each chromatographic cycle, the optical density in a portion of the eluate was similarly measured.

[b] An example for the estimate of percent bound after the third elution is as follows: $[(OD_6)/(OD_6 + OD_3)] \times 100$, where $OD_6$ and $OD_3$ are optical density values at 254 nm in the eluate obtained after the sixth and third elutions, respectively.

[c] Rechromatography of the water eluate on fresh oligo(dT)-cellulose resulted in a 95% recovery of the RNA released in the sixth water elution, which in turn was 8.9% of the original RNA.

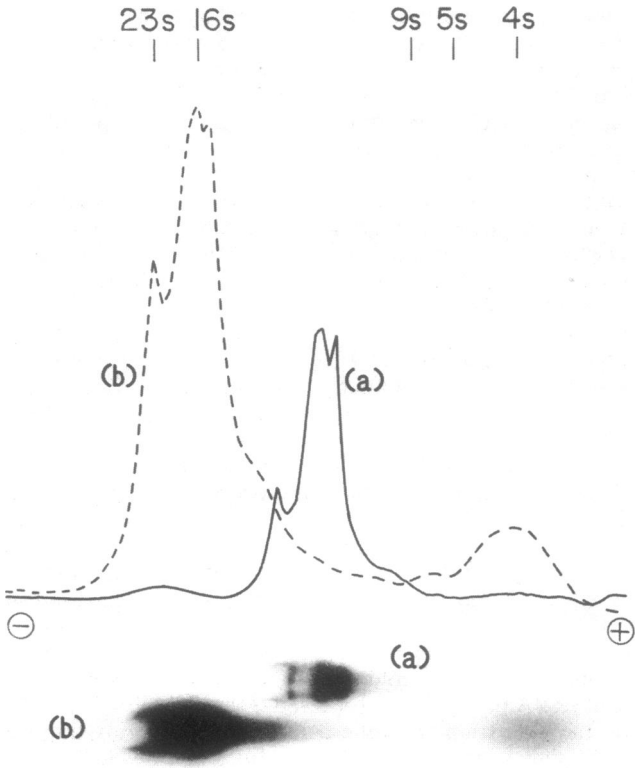


FIG. 1. Urea-polyacrylamide gel electrophoretic analysis of $^{32}$P-labeled RNAs isolated and purified from *R. rubrum*. Total RNA purified from cells was fractionated on an oligo(dT)-cellulose column as described in the text. After electrophoresis the gel was exposed to Kodak X-Omat film for 18 h. Lanes a and b contained poly(A)$^+$ and poly(A)$^-$ RNA samples, respectively. The densitometric scan was taken of the autoradiogram. The direction of electrophoresis was toward the anode.

DNA, 5S rRNA, tRNA (35, 41), heparin, and polysaccharides.

The results of the analysis of fractions unbound or bound by oligo(dT)-cellulose on denaturing gel are displayed in Fig. 1. Four bands in the resin-bound components fractionated according to their molecular weights between 16S rRNA and 9S globin mRNA, which were run as markers. Densitometric tracing of the radioautogram and its subsequent gravimetric analysis showed that the putative poly(A)$^+$ RNAs 1 through 4 accounted for approximately 5, 28, 55, and 12% of the total peak area, respectively. It should be noted that neither visual inspection of the autoradiogram nor the scrutiny of its densitometric trace revealed trace components that might have been contaminants.

The oligo(dT)-cellulose flow-through fraction contained the usual sets of RNAs that are expected of a procaryote, ranging from 23S to diffuse 4S tRNA at the bottom of the radioautogram (Fig. 1), with the labeling patterns showing obviously varied intensities.

**Separation of the poly(A)$^+$ residues from poly(A)$^+$ RNAs.** To determine the length of the poly(A)$^+$ regions in RNA, a sample of the oligo(dT)-cellulose-bound material was digested with a combination of both pancreatic A and $T_1$ RNases, processed as described above, and then subjected to chromatography on a small column of the resin. After elution and precipitation, the nuclease-resistant material was analyzed (Fig. 2) on a denaturing gel along with a synthetic poly(A) marker containing a polydisperse population with lengths ranging from 160 to 16 nucleotides (Fig. 2). A major band corresponding to about 80 nucleotides was preceded by one band and followed by two minor bands. The slowest component had an approximate length of 100 nucleotides and ran slightly faster than 5S rRNA, which was also used as marker. At the bottom end of the gel (Fig. 2), the fastest-moving poly(A)$^+$ sequence corresponded to a length larger than 16 nucleotides relative to the marker, as revealed by a

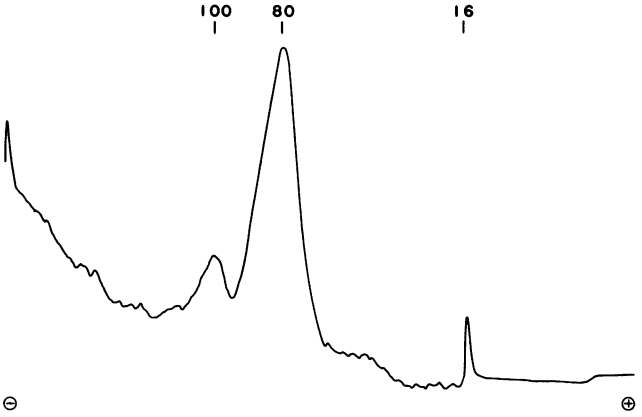


FIG. 2. Urea-polyacrylamide gel electrophoretic separation of the poly(A)$^+$ sequences purified from the poly(A)$^+$ RNA fractions after combined digestions with pancreatic A and $T_1$ nucleases. The poly(A) sequences were purified further by fractionation on a second oligo(dT)-cellulose column as described in the text. The gel, which had been polymerized from 15% acrylamide, was exposed to X-ray film for 12 h, and the autoradiogram was scanned. Standard poly(A) markers were run in a neighboring lane, and the positions were located by a scan.

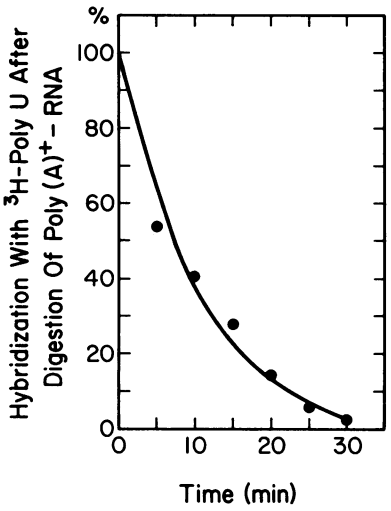


FIG. 3. Extent of hybridization of poly(A)$^+$ RNA with [$^3$H]polyuridylate after digestion with snake venom phosphodiesterase for the indicated times. The degree of hybridization in a 5.0-μl portion from the undigested sample was measured after processing as described in the text; the hybrids contained 1,100 dpm. This value corresponds to 100% at zero time and is the zero time control.

scan of the autoradiogram. Taken together, these findings establish that about 90% of the poly(A)$^+$ fragments in this procaryote contain 100 to 80 nucleotide residues.

**Nucleotide composition of the nuclease-resistant fragments.** As further confirmation of the presence of poly(A)$^+$ sequences in the oligo(dT)-cellulose-retained material, the nuclease-resistant portion of these $^{32}$P-labeled molecules was isolated by rechromatography on a fresh oligo(dT)-cellulose column. High-voltage electrophoretic analysis of this nuclease-resistant fraction after alkaline hydrolysis showed it to be almost entirely composed of adenylic acid (data not shown).

**Location of poly(A) tracts.** The poly(A)$^+$ RNA was digested with snake venom phosphodiesterase, an exo enzyme which cleaves in the 3′ → 5′ direction. Portions were taken at different times and hybridized with [$^3$H]polyuridylate as a measure of the poly(A) content of the substrate. A decrease in hybridizability commenced from zero time and progressed rapidly to almost zero hybridization (Fig. 3), establishing that the major poly(A) sequences are located at the 3′-OH ends of the poly(A)$^+$ RNA species.

**Enhanced in vitro incorporation of amino acids into hot trichloracetic acid-insoluble products by poly(A)$^+$ RNA.** To define the biological function of the poly(A)$^+$ RNAs purified from this procaryote, a heterologous, cell-free system derived from wheat germ was programmed with both RNA fractions. Poly(A)$^+$ RNA stimulated the rate of incorporation of labeled amino acid into hot trichloroacetic acid-insoluble products by approximately 22-fold in a 30-min interval when compared with the effect of the poly(A)$^-$ RNA, although the amount of this latter RNA used was 10 times that of the former (Fig. 4). Incorporation of amino acids was almost linear for 60 min. The extent of incorporation in 90 min was linear with poly(A)$^+$ RNA up to 1.5 μg (data not shown). The slight stimulation of labeled leucine incorporation above the background by the poly(A)$^-$ RNA confirmed that the poly(A)$^+$ species in this photosynthetic procaryote are mRNA molecules.

**Products of poly(A)$^+$ RNA- and poly(A)$^-$ RNA-primed translation.** The polypeptides resulting from the incorporation of [$^3$H]leucine in translations in the presence of poly(A)$^-$ and poly(A)$^+$ RNA were compared after SDS-polyacrylamide gel electrophoresis (Fig. 5). In the presence of the latter, one prominent 52,000-dalton labeled peptide could be precipitated by antibodies to the dimeric rubisco from *R. rubrum* (Fig. 5) and accounted for 6.5% of the total translation products. No labeled translation products could be detected in the incubation mixture programmed with poly(A)$^-$ RNA under comparable conditions of radioautography after SDS-polyacrylamide gel electrophoresis.

## DISCUSSION

Evidence presented in this paper establishes that poly(A)$^+$ sequences exist in oligo(dT)-cellulose-bound species of RNAs in *R. rubrum* grown photosynthetically on butyrate-bicarbonate. This fraction comprised ca. 9% of the bulk RNA and could be further separated electrophoretically into four species. Since the major poly(A)$^+$ RNA components overlap the rRNA region, the exact determination of any contamination is difficult. However, it is well established that the resin used in these experiments has negligible capacity for nonspecific binding (2, 38). Additionally, it has been known for some time that heating an RNA sample followed by rapid cooling and three passages through oligo(dT)-cellulose completely eliminates rRNAs from the resin-bound material (13, 37). Analysis of the unbound RNAs suggests some disproportionality between 23S and 16S species (Fig. 1), which might be taken to indicate the

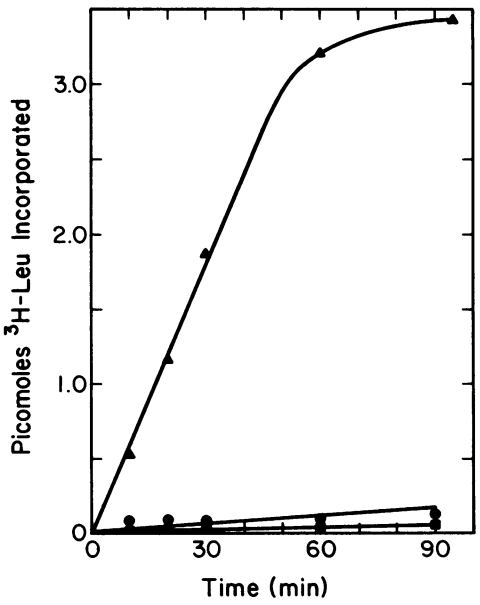


FIG. 4. Effect of *R. rubrum* RNAs on the time course of an in vitro cell-free protein-synthesizing system derived from wheat germs. The cell-free system was primed with 1.0 μg of poly(A)$^+$ RNA (▲), 10 μg of poly(A)$^-$ RNA (●), or no added RNA (■) and incubated as described in the text. At various times, 2-μl samples were processed on 3MM filter paper disks as indicated in the text. Each point reflects an average of duplicate values. The optima for pH and concentrations of $K^+$ and $Mg^{2+}$ were determined in a series of pilot experiments.

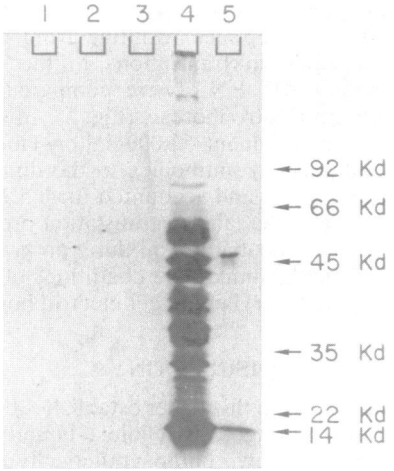


FIG. 5. Fluorogram reflecting SDS-polyacrylamide gel electrophoretic analysis of in vitro translation products. In terms of in vitro translation conditions (and subsequent treatment), the contents were as follows: no added RNA (lane 1), 10 μg of poly(A)$^-$ RNA without and with antirubisco treatment (lanes 2 and 3, respectively), 1 μg poly(A)$^+$ RNA and the resultant immunoprecipitate with antirubisco (lanes 4 and 5, respectively). Lane 6 contained the following proteins as markers: 92.5 kilodaltons, phosphorylase *b*; 66.2 kilodaltons, bovine serum albumin; 45 kilodaltons, ovalbumin; 35 kilodaltons, carbonic anhydrase; 21.5 kilodaltons, soybean trypsin inhibitor; 14.4 kilodaltons, lysozyme. The positions of the markers indicated by the arrows were revealed by staining the gel with Coomassie brilliant blue for 15 min and destaining. The gel was then soaked in En$^3$Hance solution as a first step in preparing the fluorogram. In lane 5, the radioactivity at the tracking dye front was due to (i) [$^3$H]leucine that had been bound to the antigen-antibody complex and that survived the washing and isolation procedures described in the text or (ii) small immunologically cross-reacting peptides labeled with [$^3$H]leucine (or to both).

degradation of nucleic acid during and after cellular disruption. This was not the case because two well-defined nuclease inhibitors and 2% SDS were routinely included in the buffer throughout the extraction; this combination certainly provided sufficient protection to yield poly(A)$^+$ RNA species that are highly active in in vitro translation (Fig. 5). Presumably the lower recovery of 23S rRNA reflects the fact that during 120-h culture of *R. rubrum* this species is degraded as in *E. coli* (16). Indeed, changes in growth conditions for *R. rubrum* (7) and *Rhodopseudomonas palustris* (23) invariably caused breakdown of 23S rRNA and ribosomal proteins. Additionally, in *Anacystis nidulans*, the fragmentation of 23S rRNA into $0.9 \times 10^6$- and $0.2 \times 10^6$-dalton components has been predominantly observed in cells grown beyond the log phase (8).

In spite of a report claiming the absence of poly(A)$^+$ RNA in *E. coli* (30), an increasing number of recent reports of its presence support the pioneering findings first reported from Edmonds' laboratory (9). For example, not only are these species of RNAs present, but they are heterodisperse, migrating from 20S to 4S regions when analyzed by denaturing formamide gel electrophoresis (25), and they can be varied from 15 to 1.5% as a function of the phosphate content of the growth media (38). The finding of heterodispersity in poly(A)$^+$ RNA size, independently of the techniques employed for their analyses, appears to be a general trend in procaryotes as diverse as *Bacillus subtilis* (11, 18), *Caulobacter crescentus* (27), and *R. rubrum* (21). Our data (Fig. 1) suggest that poly(A)$^+$ mRNAs in *R. rubrum* also follow this trend. By contrast, only one poly(A)$^+$ RNA of 80,000 daltons, migrating faster than 16S rRNA in 6% formaldehyde gel, has been reported to occur in induced cells of *Rhodopseudomonas capsulata* (46). The length of poly(A)$^+$ fragments in non-photosynthetic bacteria varies from 15 to 80 nucleotide residues (11, 25, 27, 33, 38). In the photosynthetic bacterium *R. rubrum*, by contrast, we observed two prominent peaks reflecting 100 and 80 nucleotide residues in addition to a minor fragment containing 16 adenylate residues (Fig. 2).

The priming of cell-free systems, derived either from eucaryotic wheat germ or *E. coli*, by poly(A)$^+$ RNAs purified from photosynthetic (21, 46) or non-photosynthetic (11) bacteria results in enhanced incorporation of radiolabeled amino acids into peptides. The present research establishes that this enhancement is 220-fold greater (on a unit mass basis) than that observed with poly(A)$^-$ RNA. An analogous comparison made with RNAs recently isolated from *B. subtilis* indicates an enhancement of about 20-fold (11). These observations imply that the poly(A)$^+$ RNA fraction is biologically functional as mRNA. Although the existence of a poly(A) sequence in mRNA molecules suggests obvious advantages, until recently very little was known about the physiological role of these sequences. Certainly they are not absolutely required for translation as evidenced by the recent findings that the partially purified messenger molecule coding for the bacteriorhodopsin in *Halobacterium halobium* is poly(A)$^-$ and yet active in wheat germ cell-free protein synthesis (6). A plausible physiological role of these sequences is that their addition to a messenger molecule, which in procaryotes is presumably posttranscriptional (38), selectively protects the transcripts against degradation by nucleases (10) and thus stabilizes these molecules (28). This conclusion offers an explanation for the differential turnover of RNAs in anaerobically cultured *Rhodopseudomonas sphaeroides*, where 40% of the total pulse-labeled RNAs were relatively stable (7, 44, 45). It may also account for enhanced longevity of mRNAs in chromosomeless *E. coli* minicells (20) and in *Myxococcus xanthus* (26).

Growth of *R. rubrum* in the butyrate-containing medium used in the present studies induces the biosynthesis of rubisco (34, 40). The present research establishes that the poly(A)$^+$ RNA species contain mRNA corresponding to this enzyme. The fractionation of these species may permit the isolation of a specific mRNA coding for this important biocatalyst. Such efforts are now in progress.

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## LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
2. **Bantle, J. A., I. A. Maxwell, and W. E. Hahn.** 1976. Specificity of oligo(dT)-cellulose chromatography in the isolation of polyadenylated RNA. Anal. Biochem. **72**:413–427.
3. **Brawerman, G.** 1974. Eukaryotic messenger RNA. Annu. Rev. Biochem. **43**:621–642.
4. **Brownlee, G. G.** 1972. Determination of sequences in RNA, p. 1–265. *In* T. S. Work and E. Work (ed.), Laboratory techniques

in biochemistry and molecular biology, vol. 3. North-Holland Publishing Co., London.
5. **Broglie, R., G. Bellemere, S. G. Bartlett, N.-H. Chua, and A. R. Cashmore.** 1981. Cloned DNA sequences complementary to messenger RNA encoding precursor to small subunit of ribulose 1,5-bisphosphate carboxylase and a chlorophyll a/b protein polypeptides. Proc. Natl. Acad. Sci. U.S.A. **78**:7304–7308.
6. **Chang, S. H., A. Majumdar, R. Dunn, O. Makabe, U. L. RajBhandary, H. G. Khorana, E. Ohtsuka, T. Tanaka, Y. O. Taniyama, and M. Ikehara.** 1981. Bacteriorhodopsin: partial sequence of mRNA provides amino acid sequence in the precursor region. Proc. Natl. Acad. Sci. U.S.A. **78**:3398–3402.
7. **Chow, C. T.** 1976. Properties of ribonucleic acids from photosynthetic and heterotrophic *Rhodospirillum rubrum*. Can. J. Microbiol. **22**:228–235.
8. **Doolittle, W. F.** 1973. Postmaturational cleavage of 23S ribosomal RNA and its metabolic control in the blue-green alga *Anacystis nidulans*. J. Bacteriol. **113**:1256–1263.
9. **Edmonds, M., and D. W. Kopp.** 1970. The occurrence of polyadenylate sequences in bacteria and yeast. Biochem. Biophys. Res. Commun. **41**:1531–1537.
10. **Gopalkrishna, Y., and N. Sarkar.** 1981. Selective resistance of bacterial polyadenylate containing RNA to hydrolysis by guanosine 3′-5′-monophosphate sensitive nuclease of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **103**:454–460.
11. **Gopalkrishna, Y., and N. Sarkar.** 1982. Characterization of polyadenylate containing RNA from *Bacillus subtilis*. Biochemistry **21**:2724–2729.
12. **Gopalkrishna, Y., and N. Sarkar.** 1982. The synthesis of DNA complementary polyadenylase containing RNA from *Bacillus subtilis*. J. Biol. Chem. **257**:2747–2750.
13. **Gorski, J., M. R. Morrison, C. G. Merkel, and J. B. Lingrel.** 1974. Size heterogeneity of polyadenylated sequences in mouse globin messenger RNA. J. Mol. Biol. **86**:363–370.
14. **Gray, E. D.** 1978. Ribosomes and RNA metabolism, p. 885–895. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
15. **Holland, M. J., and J. P. Holland.** 1978. Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-d phosphate dehydrogenase and phosphoglycerate kinase. Biochemistry **17**:4900–4907.
16. **Horiuchi, T. S., S. Horiuchi, and D. Mizuno.** 1959. Degradation of ribonucleic acid in *E. coli* in phosphorous deficient culture. Biochim. Biophys. Acta **31**:570–572.
17. **Kaeufer, N., A. Altman, and H. V. Dohren.** 1981. Isolation of highly active polysomes with polyadenylated sequences from *Bacillus bravis*. FEMS Microbiol. Lett. **12**:71–75.
18. **Karjan, P., and J. Szulmajester.** 1980. Isolation and characterization of polyadenylated RNA species from sporulating cells of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **93**:201–208.
19. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–600.
20. **Levy, S. B.** 1975. Very stable prokaryotic messenger RNA in chromosomeless *E. coli* minicells. Proc. Natl. Acad. Sci. U.S.A. **72**:2900–2904.
21. **Majumdar, P. K., and V. A. Vipparti.** 1980. Polyadenylated messenger RNAs code for photoreaction center and light-harvesting antenna polypeptides of *Rhodospirillum rubrum*. FEBS Lett. **109**:31–33.
22. **Maniatis, T., A. Jeffrey, and H. van de Sande.** 1975. Chain length determination of small double and single stranded DNA molecules by polyacrylamide gel electrophoresis. Biochemistry **14**:3787–3794.
23. **Mansour, J. D., and C. S. Stachow.** 1975. Structural changes in ribosomes and ribosomal proteins of *Rhodopseudomonas palustris*. Biochem. Biophys. Res. Commun. **62**:276–282.
24. **Marcu, K., and B. Dudock.** 1974. Characterization of a highly efficient protein synthesizing system derived from commercial wheat germ. Nucleic Acids Res. **1**:1385–1397.
25. **Nakazato, H., S. Venkatesan, and M. Edmonds.** 1975. Polyadenylic acid sequence in *E. coli* messenger RNA. Nature (London) **256**:144–146.
26. **Nelson, D. R., and D. R. Zusman.** 1983. Evidence for long-lived mRNA during fruiting body formation in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. U.S.A. **80**:1467–1471.
27. **Ohta, N., M. Sanders, and A. Newton.** 1978. Characterization of unstable poly(A)-RNA in *Caulobacter crescentus*. Biochim. Biophys. Acta **517**:65–75.
28. **Palatnik, G. W., R. V. Storti, A. K. Capone, and A. Jacobson.** 1980. Messenger RNA stability in *Dictyostelium discoideum*: does poly(A) have a regulatory role? J. Mol. Biol. **141**:99–118.
29. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA dependent translation system from reticulocyte lysate. Eur. J. Biochem. **67**:247–256.
30. **Perry, R. P., D. E. Kelley, and J. LaTorre.** 1972. Lack of polyadenylic acid sequences in the messenger RNA of *E. coli*. Biochem. Biophys. Res. Commun. **48**:1593–1600.
31. **Salser, W.** 1978. Cloning cDNA sequences: a general technique for propagating eukaryotic gene sequences in bacterial cells, p. 53–81. *In* A. M. Chakrabarty (ed.), Genetic engineering. CRC Press, Boca Raton, Fla.
32. **Sanger, F., and G. G. Brownlee.** 1967. A two-dimensional fractionation method of radioactive nucleotides. Methods Enzymol. **12A**:361–381.
33. **Sarkar, N., D. Langley, and H. Paulus.** 1978. Isolation and characterization of polyadenylate containing RNA from *Bacillus brevis*. Biochemistry **17**:3468–3474.
34. **Sarles, L. S., and F. R. Tabita.** 1983. Derepression of the synthesis of D-ribulose 1,5-bisphosphate carboxylase-oxygenase from *Rhodospirillum rubrum*. J. Bacteriol. **153**:458–464.
35. **Scragg, A. H., and C. F. Thurston.** 1975. Characterization and messenger activity of poly(A)-containing RNA from *Chlorella*. J. Gen. Microbiol. **89**:155–162.
36. **Shiness, D., and J. E. Darnell.** 1973. Polyadenylic acid segment in mRNA becomes shorter with age. Nature (London) New Biol. **241**:265–268.
37. **Singer, R. H., and S. Penman.** 1973. Messenger RNA in HeLa cells: kinetics of formation and decay. J. Mol. Biol. **78**:721–730.
38. **Srinivasan, P. R., M. Ramanarayanan, and E. Rabbani.** 1975. Presence of polyriboadenylate sequences in pulse-labeled RNA of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **72**:2910–2914.
39. **Storrø, I., and B. A. McFadden.** 1981. Glycolate excretion by *Rhodospirillum rubrum*. Arch. Microbiol. **129**:317–320.
40. **Tabita, F. R., and B. A. McFadden.** 1974. D-Ribulose 1,5-bisphosphate carboxylase from *Rhodospirillum rubrum*. I. Levels, purification and effects of metallic ions. J. Biol. Chem. **249**:3453–3458.
41. **Verma, D. P. S., D. T. Nash, and H. M. Schulman.** 1974. Isolation and *in vitro* translation of soybean leghemoglobin messenger RNA. Nature (London) **254**:74–77.
42. **Verma, I. M., R. A. Firtel, H. F. Lodish, and D. Baltimore.** 1974. Synthesis of DNA complementary to cellular slime mold messenger RNA by reverse transcriptase. Biochemistry **13**:3917–3922.
43. **Watanabe, A., and C. A. Price.** 1982. Translation of mRNA for subunits of chloroplast coupling factor I in spinach. Proc. Natl. Acad. Sci. U.S.A. **79**:6304–6308.
44. **Witkins, S. S., and K. D. Gibson.** 1972. Changes in ribonucleic acid turnover during aerobic and anaerobic growth in *Rhodopseudomonas sphaeroides*. J. Bacteriol. **110**:677–683.
45. **Yamashita, J., and M. D. Kamen.** 1968. Observation on the nature of pulse-labeled RNAs from photosynthetically or heterotrophically grown *Rhodospirillum rubrum*. Biochem. Biophys. Acta **161**:162–169.
46. **Yu, P.-L., and G. Drews.** 1982. Polyadenylated messenger RNA isolated from cells of *Rhodopseudomonas capsulata* induced to synthesize the photosynthetic apparatus. FEMS Microbiol. Lett. **14**:233–236.