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The Polynucleotide Product of Poly A Polymerase from *Escherichia coli**

S. J. S. Hardy† and C. G. Kurland

ABSTRACT: Polyriboadenylic acid (poly A) polymerase from *Escherichia coli*, an enzyme found associated with the ribosomes in a crude cell extract, has been studied both in the ribosome-bound state and in a partially purified state. No differences in the enzyme activity or specificity were found between the two states.

Adenosine triphosphate (ATP) is utilized preferentially as a substrate, the incorporation of the other nucleoside triphosphates or adenosine diphosphate (ADP) into acid-precipitable material being much less under all of the conditions studied. The enzyme requires a ribonucleic acid (RNA) primer as well as the divalent cations magnesium and manganese for optimal activity. Nearest neighbor and zone centrifugation analysis show the product of enzyme activity to be a short chain of poly A attached to the 3′ terminus of the primer RNA.

Enzymes which incorporate AMP[1] into chains of polyriboadenylic acid (poly A) have been found in vertebrate tissues as well as in bacteria (Klemperer, 1963; Burdon, 1963a,b; Edmonds and Abrams, 1960, 1962; Venkataraman and Mahler, 1963; August *et al.*, 1962; Gottesman *et al.*, 1962). All of these enzymes require ATP as substrate and a ribonucleic acid (RNA) primer. Their ubiquity suggests a basic but unknown role in nucleic acid metabolism. The provocative observation by August *et al.* (1962) that the poly A polymerase of *Escherichia coli* is apparently associated with the ribosomes has prompted the present investigation to determine whether this enzyme has some function in protein synthesis or the regulation of ribosome activity.

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate; PCA, perchloric acid; A_{260} unit, unit of material which in a 1-ml volume and light path of 1 cm will have an optical density of 1 at 260 mμ; TCA, trichloroacetic acid; $E^{260}_{1\%}$, extinction coefficient of a 1% solution at 260 mμ; TSM, Tris, 0.01 M-succinic acid, 0.003 M-$MgSO_4$, 0.01 M, pH 8.0; SDS, sodium dodecyl sulfate.

One prerequisite to an understanding of the function of such an enzyme is knowledge of the mechanism of poly A synthesis. In particular, it is necessary to determine the role of the RNA primer in the reaction. The studies of Klemperer (1963, 1965) show that the product of poly A polymerase from vertebrates is a short chain of poly A attached to the 3′ terminus of the primer RNA. In contrast, August *et al.* (1962) have suggested that the polymerase from *E. coli* synthesizes poly A *de novo* even though there is an RNA primer requirement for this reaction. However, Gottesman *et al.* (1962) isolated a similar enzyme from *E. coli* which apparently synthesizes poly A in the same manner as the vertebrate enzyme, *i.e.*, by attaching short chains of poly A to the primer RNA.

The present study was undertaken to determine the mechanism of poly A synthesis by the ribosome-bound enzyme from *E. coli*. Here, a comparison was made between poly A synthesis by the partially purified enzyme and that by the ribosome-bound enzyme. The data show that the properties of the poly A polymerase are quite similar in both states and that the product is a short poly A chain attached to the 3′ terminus of the RNA primer. The absence of any significant influence of the ribosomes on the enzymatic properties of poly A polymerase has led to a study of the structural relationship between the enzyme and the ribosomes. This latter study is reported in the next paper in this series (Hardy and Kurland, 1966).

Materials and Methods

Chemicals. All radioactive compounds and all 5′-nucleotides were purchased from Schwarz Bioresearch, Inc. (Orangeburg, N. Y.). Nucleoside monophosphates were the generous gift of Dr. G. Khorana. Deacylated soluble ribonucleic acid (s-RNA) of *E. coli* B was obtained from General Biochemicals, Inc. (Chagrin Falls, Ohio). Pancreatic deoxyribonuclease, pancreatic ribonuclease, snake venom phosphodiesterase, and muramidase were obtained from the Worthington Biochemical Corp. (Freehold, N. J.).

Bacteria. E. coli B/Tl was grown with forced aeration at 37° in a glucose salts medium supplemented with 1% vitamin-free casamino acids (Difco Laboratories). The bacteria were harvested in late exponential growth phase and stored as a frozen paste at −75°. The pilot plant facility of the Biochemistry Department of the University of Wisconsin (supported by National Institutes of Health Grant FR-00214) was employed for these preparations. We are grateful to Dr. John Garver and Mr. Arthur Olson for their help.

RNA Preparations. Ribosomal RNA was prepared by the phenol extraction procedure described previously (Kurland, 1960). Partially degraded s-RNA with the terminal 5′-AMP residue removed (CCs-RNA) was prepared by a slight modification of the method of Preiss *et al.* (1961). At the end of the incubation with snake venom phosphodiesterase the reaction was stopped by two phenol extractions of the RNA instead of by heating in a boiling water bath. Otherwise the procedure was identical with that of Preiss *et al.* (1961).

Regenerated s-RNA was prepared from the partially degraded s-RNA by a modification of the method of Preiss *et al.* (1961) using alcohol precipitation of the product rather than acid precipitation. After incubation of the reaction mixture at 37° for 30 min, the reaction was stopped by immersing the tube in ice and the reaction mixture was brought to 0.02 M EDTA and 0.15 M NaCl. RNA was precipitated by the addition of two volumes of ethanol and isolated by a low-speed centrifugation. The precipitate was dissolved in 0.1 M EDTA–0.15 M NaCl, and reprecipitated with alcohol. This step was repeated two more times and the precipitate was dissolved in distilled water and stored at −20°. By this method one-third of the s-RNA molecules incorporated a terminal AMP residue, and the acid-soluble radioactivity remaining in the preparation was less than one-third of the acid-precipitable radioactivity.

Electrophoresis of Hydrolyzed RNA. An electrophoretic analysis of an alkali hydrolysate of the RNA reaction products was performed in some of the experiments; we wish to thank M. Chamberlin for instructing us in this procedure. The reaction was stopped by the addition of 3 ml of cold 3% PCA and the precipitate of protein and nucleic acid was isolated by low-speed centrifugation and washed three times with 3 ml of 3% PCA. It was then dissolved in 0.15 ml of 0.4 N KOH and incubated overnight at 37°. The hydrolysate was brought to pH 4.0 with 10% PCA. The heavy precipitate was removed by centrifugation and washed once with 0.05 ml of water. The supernatants were combined and the mixture was applied in 0.01-ml aliquots to a strip of Whatman 3MM filter paper together with appropriate marker nucleotides. Electrophoresis was carried out in 0.05 M sodium citrate, pH 3.4, with a potential gradient of 22 v/cm and a current of approximately 20 ma. After electrophoresis, these strips were dried at room temperature and cut into pieces 2 × 3.5 cm. These pieces were immersed in scintillating fluid containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (Packard Instrument Co., Inc.) in 1 l. of toluene and radioactivity was measured in a Packard Model 3003 Tri-Carb scintillation spectrometer.

Standard Enzyme Assay. The reaction mixture for the standard assay of polyriboadenylate polymerase contained in a volume of 0.2 ml: 0.5 mM [8-^{14}C]ATP (0.5 μc/μmole), 10 mM Tris, 3 mM succinic acid, pH 8.0, 10 mM $MgSO_4$, 2 mM $MnCl_2$, 1.0 A_{260} unit of *E. coli* ribosomal or s-RNA, and 4 μg of purified enzyme or 10 A_{260} units of ribosomes with associated enzyme. These mixtures were incubated at 32° for 30 min. To stop the reaction, the tubes containing the reaction mixtures were immersed in ice and 60 μg of carrier bovine serum albumin and 1 ml of cold 0.1 M KH_2PO_4 were added. Then 2 ml of cold 7.5% trichloracetic acid was added to each tube. After the tubes had been allowed to stand in ice for 10 min, they were brought to room

TABLE I: Enzyme Purification.[a]

Fraction	Preparation I			Preparation II		
	Total Enzyme Units	Total Protein (mg)	Units/ Mg of Protein	Total Enzyme Units	Total Protein (mg)	Units/ Mg of Protein
Crude extract	1,160	1,800	0.64	4,000	1,450	2.8
$(NH_4)_2SO_4$ fraction	860	580	1.5	8,900	635	14.1
Ribosome-bound enzyme	4,460	206	21.6	11,800	243	48.5
Crude enzyme	2,260	20	113	8,900	—	—
Purified enzyme	1,390	3.6	386	3,900	3.4	1,150

[a] Two different enzyme preparations were carried out as described in the text. Small aliquots of each fraction were assayed for poly A polymerase activity in reaction mixtures which contained in a volume of 0.2 ml: 10 mM Tris, 3 mM succinic acid, 0.5 mM [8-^{14}C]ATP (0.5 μc/μmole), 10 mM Mg^{2+}, 2 mM Mn^{2+}, and 1 A_{260} unit of ribosomal RNA. The standard assay procedure was carried out. The number of enzyme units in each fraction was calculated from the total volume of each fraction and the amount of AMP incorporated into acid-precipitable material by the aliquot. Protein was measured by the method of Lowry *et al.* (1961).

temperature and the precipitates were collected by filtration through a Millipore filter (HAWP 0.25–00 0.45 μ, Millipore Filter Corp.). Both the tube and the filter were washed twice with 3.5 ml of 5% TCA. The filter was glued to an aluminum planchette and dried in an oven at 60°, and the radioactivity trapped in the filter was counted in a thin-window, gas-flow counter with an efficiency of approximately 30% for ^{14}C-labeled material. The background was estimated by measuring the amount of radioactivity obtained as acid-precipitable material from a complete mixture that was not incubated. This background was usually 100 cpm or 0.3 mμmole of incorporated AMP. In all experiments this background was subtracted from the acid-precipitable counts per minute of the incubated mixture to give the counts per minute incorporated into acid-precipitable material. One unit of enzyme activity is defined as that amount which incorporates 1 mμmole of AMP residues into acid-precipitable material in 30 min at 32°.

Concentration Measurements. Protein was measured by the procedure of Lowry *et al.* (1951) with muramidase employed as a standard. RNA concentrations were estimated spectrophotometrically and an $E_{1\%}^{260m\mu}$ of 227 was used (Kurland, 1960).

Zone Centrifugation. Zone centrifugation was carried out in linear gradients of 5–20% sucrose (Britten and Roberts, 1960).

Preparation of Enzyme. Two different kinds of poly A polymerase preparations were employed for the experiments described here. (1) *Ribosome-bound enzyme* was prepared by ammonium sulfate fractionation and differential centrifugation. All operations were carried out below 5° unless otherwise stated. Frozen bacterial paste (25 g) were slowly thawed in 15 ml of TSM which contained 10 μg/ml of deoxyribonuclease (DNAase). TSM (1 ml, tenfold concentrated) was added to the bacterial suspension which was then disrupted in a French press at 15,000–20,000 psi. The extract was centrifuged for 30 min at 25,000*g*. The resulting supernatant liquid (the crude extract of Table I) was incubated with 30 μg/ml of puromycin for 15 min at 32° in order to remove nascent protein from the ribosomes. The extract was then chilled and diluted to 100 ml with TSM.

The ribosomes were partially purified by an ammonium sulfate fractionation in three successive cycles consisting of a low followed by a high concentration of ammonium sulfate. To 100 ml of the crude extract obtained in the previous step, 21 g of ammonium sulfate was slowly added and the mixture was agitated for 3 min. To the supernatant obtained from a 10-min centrifugation at 20,000*g* an additional 21 g of ammonium sulfate was added as above. The mixture was centrifuged for 10 min at 20,000*g* and the pellet obtained was dissolved in 100 ml of TSM. The second and third cycles of the ammonium sulfate fractionation were performed as described above except that the fraction precipitating between 21 and 38.5 g of ammonium sulfate was taken in the second cycle, and that precipitating between 21 and 35 g of ammonium sulfate was taken in the third cycle.

The final pellet was resuspended in a small volume of TSM to make the total volume of crude ribosomes about 25 ml. This fraction was dialyzed against 1 l. of TSM for 2 hr, against 1 l. of fresh buffer for 4 hr, and finally against 1 further l. overnight. The dialyzed fraction, designated the $(NH_4)_2SO_4$ fraction in Table I, was centrifuged for 2 hr at 150,000*g*. The ribosomal pellet was resuspended in TSM and centrifuged again at 150,000*g* for 90 min. The pellet from this final centrifugation was resuspended in TSM and the concentration was adjusted to 500 A_{260} units/ml. These ribosomes were stored in 0.5-ml fractions at −75°, and are referred to as the ribosome-bound enzyme.

(2) *Purified enzyme* was obtained by dissociating the poly A polymerase from the ribosomes. This preparative procedure is initially the same as that for the ribosome-bound enzyme; however, the pellet from the second high-speed centrifugation of the ribosome-bound enzyme preparation was resuspended in TSM containing 0.6 M ammonium sulfate and centrifuged for 3 hr at 150,000*g*. The top four-fifths of supernatant, which is designated as the crude enzyme fraction in Table I, was removed and dialyzed overnight against 2 l. of buffer which was renewed once after 4 hr. The bottom supernatant and the pellet were discarded. During dialysis of the crude enzyme a precipitate formed in the dialysis bag; this was isolated by centrifuging the dialysate at 20,000*g* for 10 min. The supernatant was discarded, and the precipitate was washed once with 5 ml of TSM. It was then dissolved in 2 ml of TSM containing 0.6 M ammonium sulfate. In later preparations the precipitate was dissolved in 2 ml of 1 M Tris–0.3 M succinic acid. Any insoluble material was removed by centrifugation and the clear solution, which is designated the purified enzyme in Table I, was divided into 0.05-ml aliquots and stored at −75°. The activity of the preparations remained constant at this temperature for several months.

The data summarized in Table I suggest that there is an inhibitor of poly A polymerase activity in the crude extract which is lost or inactivated during the purification procedure. This is concluded from the observation of a threefold increase in enzyme activity attending the purification from steps 1 to 3. The presence of this inhibitor makes it impossible to calculate an accurate value for the purification achieved. However, if it is assumed that all the enzyme activity of the crude extract is bound to the ribosomes after the second high-speed centrifugation, then the specific activity of the enzyme in the crude extract is 2.5 units/mg of protein in preparation I and 8.1 units/mg of protein in preparation II. These values are obtained by dividing the total enzyme units of the ribosome-bound enzyme by the total protein in the crude extract. Hence, the purification achieved is 150-fold in preparation I and 140-fold in preparation II. The specific activity of poly A polymerase throughout preparation I was much lower than in any other preparation. Table I also shows that approximately 30% of the ribosome-bound enzyme is recovered as the purified enzyme. The major loss of enzyme activity occurs during dialysis when the enzyme precipitates. However, activity is also lost during the centrifugation in ammonium sulfate, and nearly all the discarded fractions contain significant activity.

It is possible that some of the activity in the discarded fractions is due to polynucleotide phosphorylase. This was tested by assaying the AMP-incorporating activity of the supernatant from the first high-speed centrifugation of preparation II using [^{14}C]ADP as substrate. The reaction conditions were the same as those of the standard poly A polymerase assay. It was found that, whereas this supernatant incorporated 1400 mμmoles/30 min of AMP when ATP was the substrate, it incorporated 2200 mμmoles/30 min of AMP when ADP was the substrate. Furthermore, it has been shown (Hardy and Kurland, 1966) that centrifugation of ribosome-bound poly A polymerase in TSM effects no release of poly A polymerase while a substantial fraction of polynucleotide phosphorylase is released. It is concluded that at least part of the activity in the first two supernatants is due to the presence of polynucleotide phosphorylase.

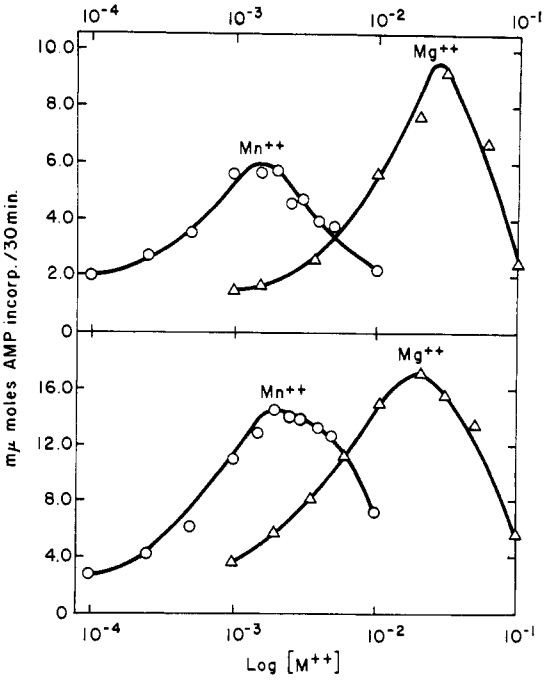

FIGURE 1: Reaction mixtures contained in a volume of 0.2 ml: 10 mM Tris, 3 mM succinic acid, 0.5 mM [8-^{14}C]-ATP (0.5 μc/μmole), 1 A_{260} unit of ribosomal RNA, and 10 mM Mg^{2+} with varying concentrations of Mn^{2+} (○), or 2 mM Mn^{2+} with varying concentrations of Mg^{2+} (Δ). Mixtures also contained (a) 2 μl of purified poly A polymerase and (b) 10 A_{260} units of ribosome-bound poly A polymerase. The standard assay procedure was followed.

Results

Properties of Enzyme Activity. It was necessary, in order to assess the functional relationship between poly A polymerase and the ribosomes as well as to facilitate the experiments described below, to carry out a comparative study of the properties of this enzyme when it is bound to the ribosomes and when it is in the purified state. Therefore, the substrate specificity, substrate concentration dependence, and divalent cation requirements of the poly A polymerase in the purified and the ribosome bound states were compared.

The data can be briefly summarized as follows. (1) Purified enzyme and ribosome-bound poly A polym-

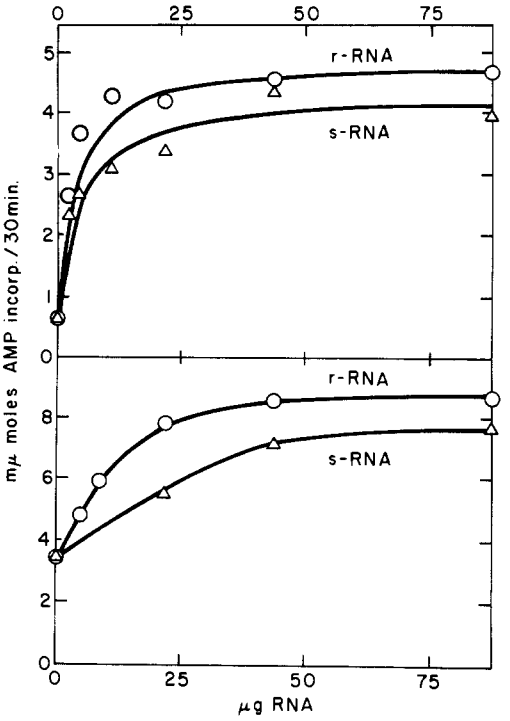

FIGURE 2: Reaction mixtures contained in a volume of 0.2 ml: 10 mM Tris, 3 mM succinic acid, 10 mM Mg^{2+}, 2 mM Mn^{2+}, 0.5 mM [8-^{14}C]ATP (0.5 μc/μmole), varying quantities of ribosomal RNA (○) or s-RNA (Δ), and (a) 2 μl of purified poly A polymerase or (b) 10 A_{260} units of ribosome bound poly A polymerase. The standard assay procedure was followed.

erase are very similar with respect to their divalent cation requirements. Both require magnesium and manganese for optimal activity (Figure 1). The optimum concentration for each ion in the presence of the other is approximately the same for both forms of the enzyme: 2 mM Mn^{2+} and 25 mM Mg^{2+}.

(2) Purified enzyme and ribosome-bound poly A polymerase preferentially utilize ATP as substrate. This result is similar to that initially described by August *et al.* (1962). Although small amounts of UMP and CMP incorporation can be obtained, it has been impossible to encourage substantial incorporation of UMP, CMP, and GMP by varying the incubation conditions with either ribosome-bound or purified enzyme.

(3) The incorporation of AMP when ADP is used as a substrate is inhibited in the presence of Mn^{2+} and a high concentration of Mg^{2+}, both of which are necessary for optimum incorporation of AMP with ATP as a substrate. In the case of the ribosome-bound enzyme the total amount of AMP incorporated when ADP is used as substrate is less than 20% of that when ATP is used as a substrate. This taken with the specific incorporation of AMP shows that the standard assay is not seriously affected by the presence of polynucleotide phosphorylase in the ribosome-bound enzyme preparation. The purified enzyme has little if any detectable polynucleotide phosphorylase activity (less than 1% of the poly A polymerase activity).

The addition of *E. coli* ribosomal RNA or s-RNA to a reaction mixture stimulates the enzyme activity of both purified and ribosome-bound poly A polymerase (Figure 2). This stimulation is more pronounced in the case of the purified enzyme. The low residual activity of the purified enzyme in the absence of added RNA can be attributed to the presence of small quantities of RNA in the enzyme preparation since treatment of one such preparation with 0.15% protamine sulfate, followed by removal of the slight precipitate formed, abolished the residual activity without affecting the activity in the presence of excess RNA. The $A_{260}:A_{280}$ ratio for the purified enzyme preparations used throughout this study was usually about 1.6 and maximum stimulation of enzyme activity by added RNA was of the order of eightfold. After protamine sulfate precipitation the $A_{260}:A_{280}$ ratio was 1.2 and maximum stimulation of enzyme activity by added RNA was greater than 50-fold. The much smaller stimulation of the ribosome-bound enzyme activity by added RNA can be attributed to a heavier contamination of RNA in the ribosome-bound enzyme preparation. The data suggest that RNA is required to prime poly A polymerase activity, but since AMP is the principle nucleotide incorporated, it is unlikely that the primer is serving as a template.

Nature of the Product. August *et al.* (1962) have shown that the product of their purified poly A polymerase preparation consists of chains of poly A which apparently terminate at the 5′ end with a 5′-phosphate monoester group. From this they suggest that chains of poly A are formed *de novo* by the enzyme. The experiments described below were initially carried out to determine whether any significant difference could be detected between the product of the purified poly A polymerase and that of the ribosome-bound enzyme.

When the ^{14}C-labeled acid-precipitable product of either purified or ribosome-bound poly A polymerase is subjected to alkaline hydrolysis and electrophoresis, the results are essentially the same (Table IIA). More than 98% of the radioactivity is recovered as 2′-(3′)-AMP with between 1 and 2% as adenosine. No significant quantities of radioactivity are found as adenosine di-, tri-, or tetraphosphate. The apparent lack of nucleotides which are characteristic of a phosphorylated 5′ end of the poly A product can be ascribed to one of two causes. First, it is possible that 5′-phosphate esters are initially present at the 5′ ends of free poly A chains, but some enzymatic activity in the poly A polymerase preparation destroys them. Alternatively the poly A chains may not be synthesized *de novo* but may be elaborated by the stepwise addition of AMP residues to the 3′ terminus of the primer RNA. In this case only the 3′ end of the product would be detectable after base hydrolysis and would be recovered as [^{14}C]adenosine.

To distinguish between these possibilities, [^{14}C]-

TABLE II: Distribution of Radioactivity in Nucleotides from Alkaline Hydrolysates of Poly A.

A. Hydrolysate of [^{14}C]Poly A Product of Poly A Polymerase[a]

Enzyme	Primer	Adenosine	Radioactivity (cpm) 2′(3′)-AMP	ADP	ATP
Purified	r-RNA[c]	190	17,600	0	20
Purified	s-RNA	220	11,480	0	25
Ribosome bound	r-RNA	75	5,845	6	0
Ribosome bound	s-RNA	240	13,580	16	0

B. Hydrolysate of [^{14}C]Poly A Product of Polynucleotide Phosphorylase[b]

Enzyme	Poly A Prepn	Adenosine	Radioactivity (cpm) 2′(3′)-AMP	ADP	ATP
Ribosome bound	I	930	8,830	780	0
None	I	1,710	15,900	1,530	0
Purified	II	55	16,530	85	12
Ribosome bound	II	40	18,850	90	19
None	II	45	18,058	70	8

[a] The products of a standard poly A polymerase reaction were subjected to hydrolysis and electrophoresis as described in Methods. [b] Radioactive poly A was added to standard poly A polymerase reaction mixtures and incubated at 32° for 30 min. It was then subjected to hydrolysis and electrophoresis. The poly A preparations were made with polynucleotide phosphorylase and were generous gifts of Violet Daniel (I) and Bernard Weisblum (II). [c] r-RNA = ribosomal ribonucleic acid.

poly A which had been prepared with polynucleotide phosphorylase was incubated in a poly A polymerase reaction mixture with and without the enzyme and was then subjected to alkaline hydrolysis and electrophoresis. In both cases the amount of radioactivity recovered as adenosine was approximately the same as that recovered as adenosine diphosphate (Table IIB). It is concluded that the 5′-monophosphate end of a free poly A chain would have been recovered by this hydrolysis and electrophoresis procedure if it had been present in the reaction mixture. Experiments were performed to test the hypothesis that the 5′ end of the poly A product is attached to the primer RNA.

Modification of the 3′ Terminus of the Primer RNA. If the mechanism of poly A synthesis is terminal addition to the 3′ end of the primer RNA, one method of detecting the 5′ end of such a product is the method of nearest neighbor analysis. Here, if an α-^{32}P-labeled ATP is used as substrate for poly A polymerase, a minor fraction of the radioactivity should appear after base hydrolysis in the 2′(3′)-mononucleotide which is derived from the 3′ terminus of the primer RNA. The remainder of the radioactivity should appear as 2′-(3′)-AMP. In addition, the number of 5′ ends of the poly A detectable by ^{32}P-labeling should be equal to the number of 3′ ends detectable by ^{14}C-labeling.

An experiment was carried out with both purified and ribosome-bound poly A polymerase using CCs-RNA as a primer to test these predictions. [^{14}C]ATP and [^{32}P]ATP were used as substrates for poly A polymerase in otherwise identical reaction mixtures. After a very short incubation to minimize the effect of contaminating diesterase activity, the acid-precipitable material was hydrolyzed and subjected to electrophoresis. With both radioactive isotopes, more than 90% of the radioactivity is found in 2′(3′)-AMP (Table III), indicating that the product is indeed poly A.

As would be expected with a primer of partially degraded s-RNA, most of the transferred ^{32}P radioactivity is found in 2′(3′)-CMP with lesser amounts in 2′(3′)-UMP and 2′(3′)-GMP. The ^{32}P found in the UMP and GMP fractions probably results from the presence of extensively degraded s-RNA and contaminating RNA in the enzyme preparation. The ratio of the radioactivity in AMP to the sum of the radioactivity transferred to CMP, UMP, and GMP gives an approximate value for the chain length of the poly A product. Similarly with the ^{14}C substrate, the chain length is equal to the ratio of the radioactivity in AMP to that in adenosine. The values for the chain length obtained with the two isotopes are approximately equal both with the purified enzyme and with the ribosome-bound enzyme (Table III). These results strongly suggest that the chains of poly A are attached to the 3′ end of the primer RNA.

Another demonstration of physical linkage of poly A to the 3′ end of primer RNA can be obtained by using as the primer s-RNA labeled in the terminal residue with [^{14}C]adenosine. This radioactive s-RNA, prepared as described in Methods, is capable of specific binding to ribosomes (Kurland, 1966), and is therefore functionally active at least in this respect. Reaction mixtures were

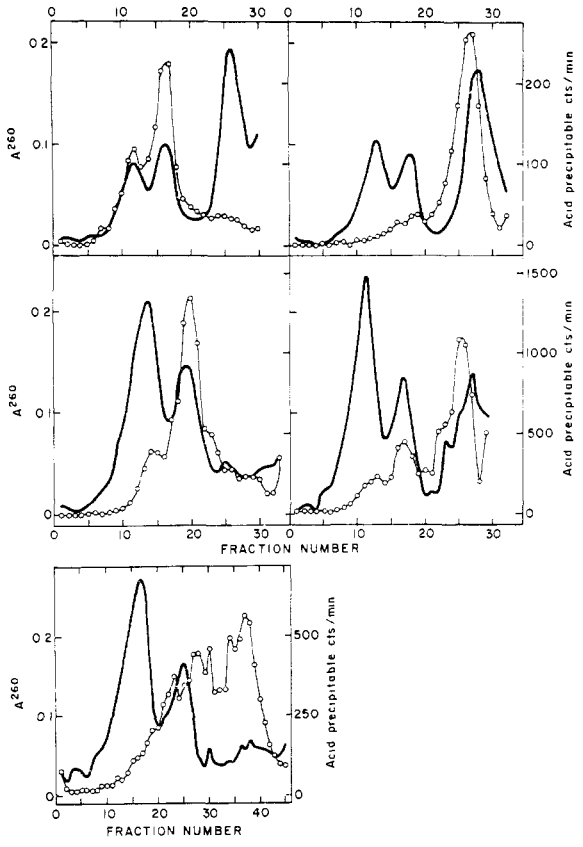

FIGURE 3: Standard reaction mixtures were made up with purified poly A polymerase in volumes of 0.2 ml. The primer was (a) 4 A_{260} units of ribosomal RNA or (b) 4 A_{260} units of s-RNA. After a 28-min incubation 4 A_{260} units of s-RNA was added to a and 4 A_{260} units of ribosomal RNA to b. After 30-min incubation the reaction was stopped by cooling in ice and 0.01 ml of 2% SDS was added. An aliquot of each mixture was removed for the standard assay and the remainder was layered on sucrose gradients containing 0.1 M Tris, 0.03 M succinic acid, and 0.1% SDS, and centrifuged for 6 hr at 36,000 rpm in a Spinco SW39 head. The A_{260} and acid-precipitable radioactivity (O———O) of each fraction were measured. Unincubated controls gave the same A_{260} pattern but no acid-precipitable radioactivity. Recovery of radioactivity was greater than 90%. The same experiment was performed with ribosome-bound poly A polymerase except that reaction mixtures were 0.6 ml and no RNA was added after 28-min incubation. Primers were (c) ribosomal RNA, (d) s-RNA, and (e) no primer. The mixtures were brought to 0.5% SDS and centrifuged for 16 hr at 25,000 rpm in a Spinco SW25 head.

made up containing [^{14}C]adenosine s-RNA, nonradioactive ATP, and poly A polymerase. After a short incubation the acid-precipitable product was subjected to the normal hydrolysis and electrophoresis procedure. All the radioactivity of the control, incubated in the absence of enzyme, was found in adenosine as expected

TABLE III: Identification of Termini of Poly A Polymerase Product.[a]

Nucleotide	Radioactivity (cpm) Purified Enzyme	Ribosome-Bound Enzyme
A. [α-^{32}P]ATP Substrate		
2′(3′)-AMP	17,600	13,120
2′(3′)-CMP	530	290
2′(3′)-GMP	45	60
2′(3′)-UMP	315	110
ADP	80	85
Unidentified peak	540	115
Ratio AMP:(CMP + GMP + UMP)	19	29
B. [8-^{14}C]ATP Substrate		
Adenosine	680	160
2′(3′)-AMP	10,920	6,240
ADP	0	5
Ratio AMP: Adenosine	16	39

[a] Reaction mixtures contained [α-^{32}P]ATP (8 μc/μmole), [8-^{14}C]ATP (2.5 μc/μmole), and 0.25 A_{260} unit of partially degraded s-RNA in 0.2 ml of reaction mixture. Incubation was for 6 min at 32°. With the [α-^{32}P]ATP substrate an unidentified peak of radioactivity always appeared between the AMP and GMP markers. No corresponding peak of radioactivity appeared with the [8-^{14}C]ATP substrate.

(Table IV). In contrast some of the radioactivity in the hydrolysate of the poly A polymerase product was present as 2′(3′)-AMP (Table IV). These results also strongly suggest that the poly A polymerase preparations attach at least a single phosphate group to the 3′ end of an s-RNA primer. They also indicate that undegraded functional s-RNA can act as a primer for this enzyme.

There is a serious objection to the above experiment. It was noted in the Methods section that the [^{14}C]-adenosine s-RNA preparation contained a significant quantity of nonacid-precipitable radioactivity, a proportion of which is probably still in the form of ATP. This ATP would act as a substrate for poly A polymerase and would be incorporated into chains of poly A. After hydrolysis it would appear as radioactive AMP and might give rise to results such as those reported in Table IV. However, a maximum estimate of the radioactivity in adenylic acid due to this artifact, based on the poly A polymerase activity of the preparations used and the quantity of nonacid-precipitable radioactivity in the [^{14}C]adenosine s-RNA, showed that less than one-tenth of the counts in 2′(3′)-AMP for the purified enzyme and less than one-third for the ribo-

TABLE IV: Terminal Modification of [^{14}C]Adenosine s-RNA.[a]

Enzyme	Adenosine	2′(3′)-AMP	ADP
No enzyme	2055	2	0
Purified enzyme	1625	297	0
Ribosome-bound enzyme	1870	73	0

[a] Standard reaction mixtures were made up in a total volume of 1 ml with nonradioactive ATP and regenerated [^{14}C]adensoine s-RNA at a concentration of 1 A_{260} unit/ml. They were incubated for 10 min at 32°. During this time the radioactivity in the regenerated s-RNA was not conserved, about 40% being lost from the tubes containing enzyme. The reaction was stopped by the addition of 3% PCA, and the product was hydrolyzed and subjected to electrophoresis as described in Methods.

some-bound enzyme can be accounted for in this way.

Physical Attachment of Poly A to Primer RNA. Although the experiments presented above suggest that there is a physical attachment of AMP residues to the 3′ end of the primer RNA, they do not rule out the possibility that only very short chains of AMP residues are attached to the primer while the bulk of the poly A is in the form of free chains having a 3′-terminal phosphate and lacking a 5′-terminal phosphate. Furthermore, each of the above experiments is open to the criticism that a combination of different contaminating enzymes other than poly A polymerase could be responsible for the observed results. Therefore, it was necessary to show that all of the poly A product of the polymerase is physically attached to the primer. This was done by a zone centrifugation analysis of the poly A formed with RNA primers having radically different sedimentation coefficients (Figure 3).

Reaction mixtures were made up with [^{14}C]ATP, purified poly A polymerase, and either ribosomal RNA (Figure 3a) or s-RNA (Figure 3b). These were incubated for 30 min at 32°. Two minutes before the end of the incubation, s-RNA was added to the reaction mixture containing ribosomal RNA, and ribosomal RNA was added to the mixture containing s-RNA. This was done to exclude the possibility that the attachment of poly A to the primer is due to a temperature-dependent adsorption. The reaction mixtures were brought to 0.2% SDS in order to free the RNA from protein, and centrifuged through sucrose gradients containing this detergent. The A_{260} and acid-precipitable radioactivity in each fraction were determined. Almost all the radioactivity sedimented with the original primer. The possibility that the poly A is noncovalently bound to the primer is excluded by the results of Moore and Asano (1966) and of Hayes *et al.* (1966) who state that, even in the presence of divalent cations, which are not present in these gradients, poly A does not bind to ribosomal RNA.

Examination of Figure 3b shows that the peak of radioactivity sedimented appreciably faster than the peak of s-RNA, as would be expected of an s-RNA molecule whose molecular weight was increased by the addition of 50 AMP residues. The same shift is not seen for a ribosomal RNA primer, probably because the relative increase in the molecular weight is much smaller and the change in the rate of sedimentation is not sufficient to be detected in this type of experiment.

A similar experiment was done for the ribosome-bound poly A polymerase (Figures 3c and d) except that the nonpriming RNA was not added at the end of the incubation. Similar results were obtained. Therefore, it is concluded that there is a physical attachment between the RNA primer and the poly A chains formed by both purified and ribosome-bound poly A polymerase. Figure 3e shows the sedimentation characteristics of the poly A formed by ribosome-bound enzyme in the absence of added primer. Clearly the contaminating RNA in the ribosome, bound enzyme is heterogeneous and has sedimentation coefficients mainly between 16 and 4 S. It is concluded that all of the poly A product of poly A polymerase in both the purified and ribosome-bound states is a chain of AMP residues linked through a phosphate ester bond to the 3′ terminus of the primer RNA.

Conclusions

The work of August *et al.* (1962) suggested that the poly A polymerase associated with the ribosomes in *E. coli* synthesizes poly A *de novo* even though an RNA primer is required. In contrast, Gottesman *et al.* (1962) have described an enzyme from *E. coli* which attaches short chains of poly A to the required RNA primer and may be identical with the one studied here. The data reported in the present paper suggest that the poly A polymerase associated with the ribosomes of *E. coli* does not synthesize free chains of poly A but attaches chains of poly A to the 3′ terminus of the RNA primer. The discrepancy between the results presented here and those of August *et al.* (1962) is at present inexplicable. However, their nearest neighbor studies showed some transfer of ^{32}P to CMP, UMP, and GMP, qualitatively similar to that reported by Gottesman *et al.* (1962) and to that reported here.

The poly A polymerase of *E. coli* is, therefore, one of a class of widely occurring enzymes which attach short chains of poly A to the 3′ terminus of an RNA primer. Their ubiquity suggests that such enzymes play an important role in nucleic acid metabolism. However, two questions must be answered before it will be possible to determine what this function is. First, it is necessary to determine which RNA fraction is the *in vivo* primer of the poly A polymerase. Second, it is necessary to determine whether or not the association of poly A polymerase with the ribosomes of *E. coli*

is fortuitous. The next paper in this series is concerned with this latter question; data will be presented which strongly suggest that there is no functional relationship between the poly A polymerase and the ribosomes.

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The Relationship between Poly A Polymerase and the Ribosomes*

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ABSTRACT: The structural and functional relationship between polyriboadenylic acid (poly A) polymerase and the ribosomes of *Escherichia coli* was studied. Four experimental criteria to decide whether or not an enzyme is a structural element of the ribosomes were applied. These are: (1) that there is some functional relationship between the enzyme and the ribosomes; (2) that the enzyme remains associated with the ribosomes under conditions which remove strongly adsorbed supernatant proteins and yield active ribosomes; (3) that the enzyme is specifically associated with either the 30- or 50S ribosomal subunit; (4) that the number of ribosomal binding sites for the enzyme is well defined. Poly A polymerase fails to meet each of these criteria, and it is concluded that the enzyme is an adsorbed contaminant of the ribosomes. A survey of other putative ribosomal enzymes suggests that none of them has been convincingly identified as a structural element of the ribosomes and, therefore, that they may also be contaminants.

It is as yet impossible to assign a function to any particular molecular component of the ribosomes. Indeed, the task is a formidable one since the ribosomes of *Escherichia coli* contain between 20 and 40 electrophoretically distinct protein components (Waller and Harris, 1961; Waller, 1964; E. C. Cox and J. G. Flaks, personal communication, 1966), as well as at least two ribonucleic acid (RNA) molecules (Littauer and Eisenberg, 1959; Kurland, 1960). In addition to these physically separable components there have been a number of reports of ribosomal enzymes (Elson, 1958, 1959; Bolton and McCarthy, 1959; Matheson and Tsai, 1965; Tsai and Matheson, 1965; Wade and Lovett, 1961; August *et al.*, 1962; McCorquodale, 1963). If some of the ribosomal proteins could be shown to have an enzymatic activity which could be assayed independently of the ribosome function in polypeptide

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