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Ribonucleic Acid Dependent Deoxyribonucleic Acid Synthesis by *Escherichia coli* Deoxyribonucleic Acid Polymerase I. Characterization of the Polymerization Reaction†

Elizabeth C. Travaglini* and Lawrence A. Loeb

ABSTRACT: The RNA dependent DNA polymerization activity of *Escherichia coli* polymerase I is an intrinsic part of the enzyme molecule as shown by several methods (gel filtration, isoelectric focusing) and a requirement for enzyme-bound Zn^{2+}. The DNA product of the reaction is covalently bound to the RNA initiator-template complex. The stoichiometry between the enzyme and the RNA template has been investigated and the mole ratio of the two was found to be critical for extended DNA synthesis and for the ultimate size of the product. The *"de novo"* poly[d(A-T)]·[d(A-T)] synthesis which occurs concurrently with the RNA dependent DNA reaction was minimized by using a high concentration of Mg^{2+} (8 mM) in the reaction mixture. Our evidence indicates that the polymerase has a double-stranded RNA endonuclease activity which produces 3′-OH termini; this enables the polymerase to create additional points of initiation for DNA synthesis on RNA molecules containing double-stranded regions.

Now that it is possible to obtain DNA copies of unique sequences of RNA, new and powerful methods are available for the analysis of viral replication, integration, and function as well as for the investigation of RNA metabolism in cellular differentiation. These DNA copies have been synthesized from RNA templates using RNA dependent DNA polymerases ("reverse transcriptases") from animal tumor viruses, particularly avian myeloblastosis virus, AMV (Temin and Baltimore, 1972). We and others have shown that natural RNAs can also be faithfully copied by *Escherichia coli* DNA polymerase I (Pol I)[1] (Loeb *et al.*, 1973; Modak *et al.*, 1973). This finding has recently been confirmed by Gulati *et al.* (1974) who found that the DNA copies of rabbit globin mRNA synthesized by Pol I were indistinguishable by hybridization kinetics from those synthesized by AMV polymerase. Highly purified Pol I is available in large quantities and appears to be an ideal tool for copying a variety of RNA templates. The DNA copies can then be isolated and used as templates for repetitive net synthesis. This procedure could provide large amounts of specific complementary DNAs for the study of the role of a particular RNA in cellular metabolism.

It should be noted that a comparison of the specific activities of purified Pol I and the DNA polymerases from tumor viruses indicates that they are all more active in copying DNA templates than RNA templates (Loeb, 1974). The efficiency of Pol I in copying RNA templates is similar to that of the "reverse transcriptases." However, Pol I copies "activated" DNA 500–1000 times more efficiently than rRNA (Loeb *et al.*, 1973).

Hitherto, even though most of the sequences in the RNA template appear to be copied with viral RNA (Garapin *et al.*, 1973; Tavitian *et al.*, 1974), the DNA copies have been small molecules compared to the molecular size of the RNA template no matter whether the polymerase used is Pol I or a "reverse transcriptase" (Gulati *et al.*, 1974).

To better understand how this is possible, we have analyzed in detail the RNA dependent DNA polymerization reaction using Pol I. To do this, we have used 28S rRNA as a template since, unlike most other RNAs investigated, no added oligonucleotides, which might make the interpretation of the reaction more complex, were necessary to initiate the polymerization reaction. We have already shown in a previous paper (Loeb *et al.*, 1973) that the requirements for catalysis of polymerization using Pol I with an RNA template are essentially identical with those using Pol I with a DNA template.

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[1] Abbreviation used is: Pol I, *E. coli* DNA polymerase I.

In the present work, the RNA dependent polymerization activity has been proven to be an integral part of Pol I. The stoichiometry between the enzyme and the template has been determined and the role of the nuclease activities, which are an intrinsic part of Pol I, in the formation of the DNA product has been elucidated. Finally, the nature of the DNA product during the course of synthesis has been established.

Materials and Methods

E. coli DNA Polymerase I. The enzyme was purified by the method of Jovin *et al.* (1969), including extensive phosphocellulose chromatography and "Sephadex G-100" gel filtration. The fraction used in these experiments was that used by Springgate *et al.* (1973). The enzyme was estimated to be 99% homogeneous by electrophoresis on polyacrylamide gels in a variety of conditions and had a specific activity with maximally "activated" calf thymus DNA (Fansler and Loeb, 1974) of 24,000 units/mg of protein (Kornberg units) (Jovin *et al.*, 1969).

Deoxynucleotides. Unlabeled nucleotides were purchased from Calbiochem; ^{3}H-labeled and ^{32}P-labeled nucleotides were obtained from New England Nuclear Corp.

RNA. rRNA was purified from third instar *Drosophila melanogaster* larvae as outlined by Tartof and Perry (1970) and from L-cells by the method of Perry and Kelley (1968). The [^{14}C]RNA (28 S) from L-cells was obtained from cells (5.8×10^5/ml) which had been grown in the presence of 0.1 μCi/ml of [^{14}C]uridine (56.7 Ci/mol) for 43 hr. The 28S RNA component from each source was further purified on 5–25% (w/w) linear sucrose gradients containing 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 100 mM NaCl, and 0.5% sodium dodecyl sulfate. Centrifugation was at 21,000 rpm in an SW 27 rotor for 16 hr at 20°. The specific activity of the ^{14}C-labeled L-cell 28S rRNA was 15,000 dpm/μg of RNA.

Polymerase Assays. With an RNA template, the enzyme activity was determined in a 0.05-ml reaction mixture containing 60 mM Tris-HCl (pH 7.0), 5 mM dithiothreitol, 8 mM $MgCl_2$, 100 μM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (400 dpm pmol^{-1}), 2.4 pmol of 28S rRNA, and 10 μl of the appropriate enzyme fraction. The reaction mixtures were incubated for 60 min at 37°. The acid-insoluble product was precipitated with 0.1 ml of 1 M $HClO_4$ containing 0.02 M sodium pyrophosphate using 0.1 ml of DNA (1 mg/ml) as carrier. Then, the precipitate, dissolved in 0.5 ml of 0.2 M NaOH–0.05 M sodium pyrophosphate, was reprecipitated with acid, collected on glass fiber filters, and counted by liquid scintillation spectroscopy. Activity is expressed as the amount of nucleotide incorporated by each fraction during the indicated period of incubation.

When DNA was used as template, the polymerase activity was determined in a 0.30-ml reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, 50 μM each of dATP, dGTP, dCTP, and [^{32}P]dTTP, 0.6 μM of "activated" calf thymus DNA(P), and 5 μl of the appropriate enzyme fraction. The reaction mixtures were incubated for 10 min at 37°; the acid-insoluble product precipitated with acid in the presence of carrier DNA and then was collected on glass fiber filters and counted by liquid scintillation spectroscopy (Loeb, 1969).

RNA Concentrations. The 28S RNA concentrations are expressed as moles of intact 28S molecules and not as moles of nucleotide or nucleotide-phosphorus. Based on the fact that 28S RNA has a molecular weight of 1.7×10^6, 1 μg of RNA is equivalent to 0.6 pmol.

Pol I Concentrations. The molecular weight of Pol I is 1.09 $\times 10^5$ (Jovin *et al.*, 1969); 1 μg of homogeneous Pol I is equivalent to ~10 pmol.

E/R Ratio. E/R is the molar ratio of Pol I to 28S RNA that is present in the reaction mixture.

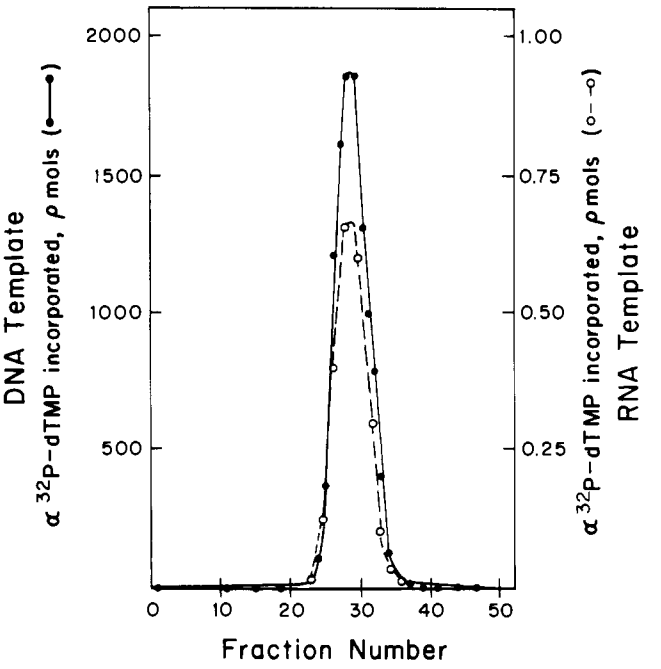

FIGURE 1: Gel filtration of *E. coli* polymerase I in Sephadex G-100. Purified *E. coli* DNA Pol I (500 μg) was applied to the bottom of a 1 × 100 cm column containing G-100 Sephadex. The Sephadex had previously been washed with bovine serum albumin (Loeb, 1969) and equilibrated with buffer (0.1 M Tris-HCl (pH 7.5)–0.1 M KCl). The column was eluted by upward flow with the same buffer at a flow rate of 10 ml/hr; 5-ml fractions were collected. Polymerase activity was assayed across the peak using both a DNA template and an RNA template. See Methods for assay procedures.

Results

DNA and RNA Dependent Pol I Activity in a Single Molecule. The RNA dependent DNA polymerase activity of Pol I appears to be an integral part of the enzyme as determined by several independent criteria. To ensure that the RNA dependent DNA polymerization activity was not due to an enzyme which had a high turnover number and which might escape detection in the 1% protein contaminating the Pol I preparation, the two polymerizing activities of Pol I were compared using two different methods of separation: filtration on Sephadex G-100 which separates proteins primarily on the basis of molecular size, and isoelectric focusing which separates the proteins on the basis of their isoelectric point. When Pol I was subjected to gel filtration, its RNA dependent and DNA dependent activities were found in the same fractions and the ratio of the two activities remained constant across the enzyme peak (Figure 1). A very similar result was obtained when the enzyme was subjected to isoelectric focusing on a pH stabilized glycerol gradient (Figure 2). The absolute incorporations of nucleotide cannot be compared for the RNA and DNA templates since the concentration of the RNA template is not at saturation in these experiments. The slight secondary peak observed may be due to the appearance of the large fragment of Pol I (Klenow and Henningsen, 1970; Setlow *et al.*, 1972) resulting from the cleavage of the enzyme during isoelectric focusing. This secondary peak also responds to both DNA and RNA templates, suggesting that the large fragment can copy both templates.

To further substantiate that the two template dependent activities were functions of the same enzyme, we measured the

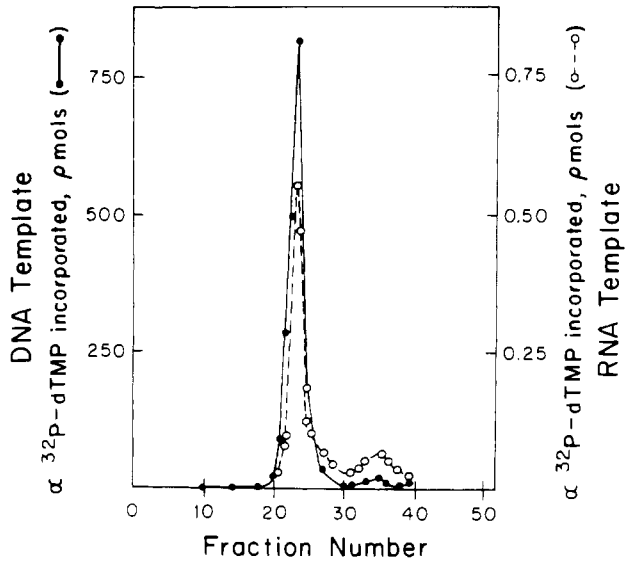

FIGURE 2: Isoelectric focusing. *E. coli* DNA Pol I (100 μg) was electrofocused on a minicolumn at 4°. The apparatus consisted of 5-ml plastic pipettes (Falcon 7529) connected by Tygon tubing. One limb contained 1.0 ml of 60% sucrose solution over which 5 ml of a 10–40% linear glycerol gradient containing 10 mM dithiothreitol and 0.8% ampholine (pH range 3–10) was added. Then a 2% ethylenediamine solution was gently layered over the glycerol gradient and the limb connected to the cathode. The other limb was filled with 0.2 M phosphoric acid and served as the anode. Platinum electrodes were used at both termini. The column was prerun at 600 V for 1 hr to establish a pH gradient. The polymerase contained in 20% glycerol was delivered through a fine Teflon tubing to the middle of the pH–glycerol gradient. The enzyme sample was then electrofocused for 5 hr at 600 V after which 0.1-ml fractions were collected from the bottom of the gradient. The polymerase activity was determined by using an aliquot of each fraction as given in Methods.

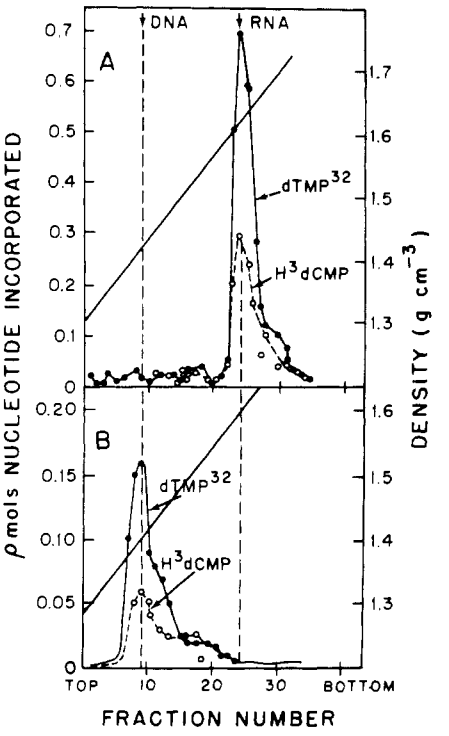

FIGURE 3: Equilibrium sedimentation in Cs_2SO_4 of the reaction product. The reaction mixture which is described in Methods was used with *Drosophila* 28S RNA as template, [α-^{32}P]dTTP and [^{3}H]dCTP as labeled precursors, and an enzyme to RNA ratio (E/R = 9). (A) RNA–DNA product was heated for 10 min at 60° in 1 ml of 0.05 M KCl, pelleted through CsCl to remove protein, and then mixed with Cs_2SO_4 containing 0.5% Sarkosyl to a final density of 1.550 g cm^{-3} and centrifuged at 40,000 rpm for 72 hr in a Spinco SW 50.1 rotor. Poly[d(A-T[^{3}H])]·poly[d(A-T[^{3}H])] and L-cell ^{14}C-labeled 28S RNA were used as markers and run on a separate gradient in the same rotor. Fractions containing 8 drops each were collected from the top of the gradient into tubes. The radioactivity in each fraction was determined as described in Methods. (B) Same as (A) except that the protein-free product was hydrolyzed in 0.5 M NaOH for 5 min at 100° before being mixed with Cs_2SO_4 containing 0.5% Sarkosyl. The densities of DNA and RNA are indicated by the dotted lines.

RNA dependent polymerization activity using the "zinc-free" apoenzyme. Springgate *et al.* (1973) have shown that Pol I contains 1 g-atom of Zn/mol of enzyme, and that when Zn is removed from the enzyme, an apoenzyme results. The preparation of apoenzyme used in these experiments contained 0.06 g-atom of Zn/mol of enzyme which may represent contamination with the holoenzyme. This preparation of apoenzyme had a DNA dependent polymerase activity, using activated DNA as a template, 7% that of the holoenzyme. Using 28S RNA as a template, we compared the RNA dependent activity of the apoenzyme with that of the holoenzyme. We found that the activity of the apoenzyme was 12% that of the holoenzyme in a 1-hr incubation at 37°. The difference between a 7% activity with DNA and a 12% activity with RNA is not considered significant because the incubation period using RNA as a template may be of sufficient length to allow any contaminating Zn^{2+} present in the reaction mixture (approximately 5 μM) to combine to some extent with the apoenzyme and so increase its activity.

Relationships between Enzyme and Template. Having ascertained that Pol I is indeed the enzyme catalyzing DNA polymerization when an RNA template is used, it became important to investigate the relationships between the enzyme and the template. Ribosomal 28S RNA was used for our studies since unlike most other RNAs investigated, no added oligonucleotides were necessary to initiate the polymerization reaction. Single RNA molecules which may serve as both initiator and template for Pol I should facilitate our understanding of the relationships between template and enzyme during the reaction.

Equilibrium sedimentation in neutral Cs_2SO_4 of the reaction product after 1-hr incubation indicates that the DNA product was covalently bound to the 28S RNA template. As can be seen in Figure 3A, the DNA product equilibrates at the density corresponding to that of RNA in Cs_2SO_4, suggesting that the bulk of the molecule is the RNA template. However, when the RNA template is removed from the product by alkaline hydrolysis, the banding pattern in neutral Cs_2SO_4 is different, and the radioactive product, free of RNA, bands at the buoyant density of DNA (Figure 3B).

Further evidence for covalent binding of the DNA product to the RNA being copied was obtained from the experiment shown in Figure 4. A phenol extracted reaction product from a 2-hr incubation using L-cell ^{14}C-labeled 28S RNA was completely denatured with 90% dimethyl sulfoxide (we obtained the same results with 90% formamide) (Strauss *et al.*, 1968) and banded in a neutral Cs_2SO_4 gradient containing 10% dimethyl sulfoxide. Under these denaturing conditions, most of the DNA bands with the RNA template and thus must be covalently bound to the RNA.

A time course for the reaction at various concentrations of enzyme is shown in Figure 5. When the concentration of RNA is constant (0.75 pmol), the rate of nucleotide incorporation into an acid-insoluble product is proportional to the amount of enzyme. Similarly, nucleotide incorporation is proportional to the RNA concentration (Figure 6). Saturation with respect to RNA template is approached at the higher concentrations of RNA in the presence of limiting amounts of enzyme. Since we were interested in a large amount of product, we found it expe-

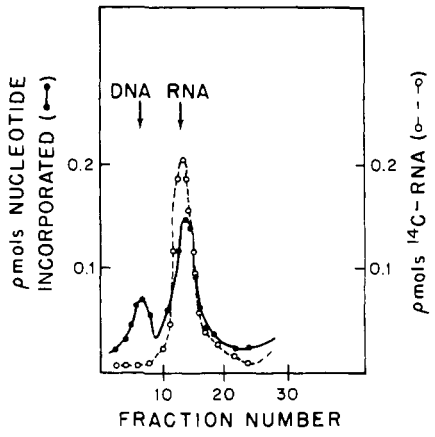

FIGURE 4: Equilibrium sedimentation in Cs_2SO_4—10% dimethyl sulfoxide of the denatured reaction product. The reaction mixture which is described in Methods was used with L-cell ^{14}C-labeled 28S RNA as template, [^{3}H]dCTP as the labeled precursor, an E/R ratio = 9, and an incubation period of 2 hr. The reaction product was extracted with phenol-chloroform and precipitated with 100 μg of yeast sRNA in 2 volumes of ethanol overnight at −20°. The precipitate was dissolved in 0.01 M Tris-HCl (pH 7.4)-1 mM EDTA and then denatured with dimethyl sulfoxide (final concentration, 90%) held for 1 min at 60° (Strauss *et al.*, 1968). Concentrated Cs_2SO_4 was added to a final density of 1.550 g cm^{-3} (the final concentration of dimethyl sulfoxide in the gradient was 10%) (Williams and Vinograd, 1971) and the mixture was centrifuged at 40,000 rpm for 72 hr in a Spinco SW 50.1 rotor. The gradient was fractionated from the top and the radioactivity of each fraction was determined as described in Methods. L-cell ^{14}C-labeled 28S RNA and poly[d(A-T[^{3}H])]·poly[d(A-T[^{3}H])] were used as markers and run in a similar gradient in the same rotor.

dient to use a high molar ratio of enzyme to RNA, E/R, for most of our experiments. The RNA concentrations in the work to be described are expressed as moles of intact 28S RNA molecules and not as moles of nucleotide or nucleotide-phosphorus, since each intact 28S RNA molecule is presumed to have only one 3′-OH initiator end.

Prevention of "de novo" Poly[d(A-T)] Synthesis during Long Incubation Periods. Pol I is known to catalyze the formation of a d(A-T) (poly[d(A-T)]·poly[d(A-T)]) polymer in the absence of a template after a lag period of 1-3 hr (Schachman *et al.*, 1960). Once started, the synthesis proceeds very rapidly.

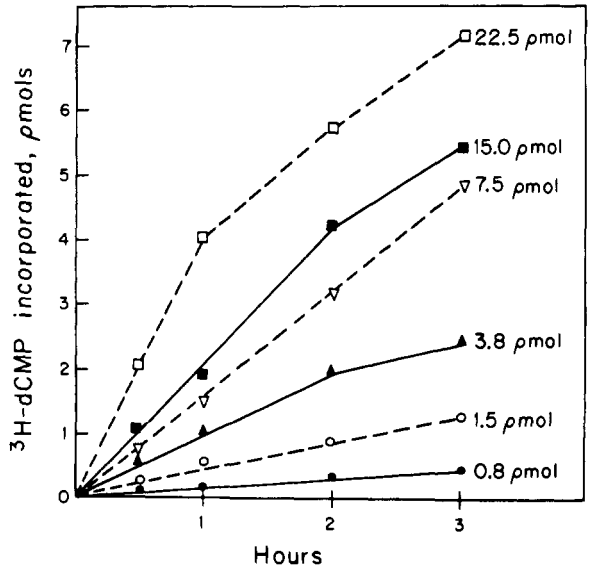

FIGURE 5: Time courses for deoxyribonucleotide incorporation into a DNA product at various enzyme concentrations. The assay conditions were the same as those described in Methods, except that the radioactive nucleotide was [^{3}H]dCTP, the amount of *Drosophila* 28S RNA was 0.75 pmol, and the amount of enzyme was varied as indicated.

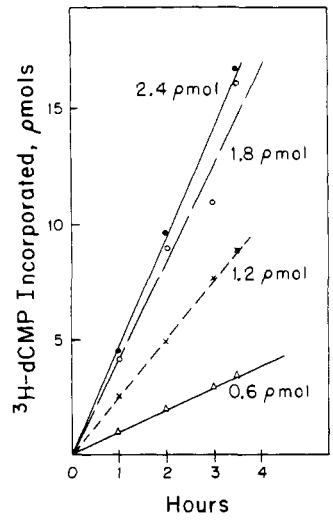

FIGURE 6: The effect of the RNA template concentration on the incorporation of deoxyribonucleotides into DNA. The assay conditions were the same as those described in Methods, except that [^{3}H]dCTP was the labeled nucleotide. The concentration of Pol I was 22 pmol, and that of the *Drosophila* 28S RNA is as indicated for each time course.

Neither deoxycytidine nor deoxyguanosine triphosphate is necessary for d(A-T) synthesis, nor are they incorporated into the product. Since this type of rapid *"de novo"* d(A-T) synthesis, once started, would quickly deplete the dATP and dTTP substrates present in the reaction mixture, and since prolonged incubation periods are desirable to synthesize substantial amounts of DNA from an RNA template, it was important to prevent or minimize *"de novo"* d(A-T) synthesis. We found that the concentration of Mg^{2+} ions in the reaction mixture affects the lag period involved in *"de novo"* synthesis. By using a double label, [^{32}P]dTTP, which is incorporated into both poly[d(A-T)] and DNA complementary to RNA templates, and [^{3}H]dCTP, which is incorporated only into the DNA polymerized from the RNA template, we found that concentrations of Mg^{2+}, 6 mM or greater, increased the lag period for *"de novo"* synthesis to 3 hr or more, whereas at 4 mM Mg^{2+} the lag period decreased to 1 hr or less. No concentration of Mg^{2+} utilized enabled us to increase the lag period to 4 hr. The effect of Mg^{2+} is shown in Table I. The ratio of thymidine to cytidine incorporation, T/C, in a complementary DNA copy of Drosophila 28S RNA should be ~1.3; this ratio of T/C was observed for all Mg^{2+} concentrations 6 mM or greater and for incubation periods up to 3 hr. However, after a 4-hr incubation, a much greater amount of dTMP than dCMP is incorporated at all Mg^{2+} concentrations except at 18 mM. A CsCl gradient analysis of the products of a 4-hr incubation indicated that poly[d(A-T)] is synthesized. Thus, by using high concentra-

TABLE I: Effect of Mg^{2+} Concentration on the *"de novo"* Synthesis of d(A-T).

[Mg^{2+}] (mM)	Ratio of dTMP/dCMP Incorporated (Incubation, hr)			
	1	2	3	4
4	1.6	3.5	4.9	6.9
6	1.2	1.3	1.2	1.7
8	1.2	1.3	1.4	2.0
10	1.3	1.4	1.2	2.0
14	1.4	1.4	1.3	1.7
18	1.3	1.4	1.4	1.3

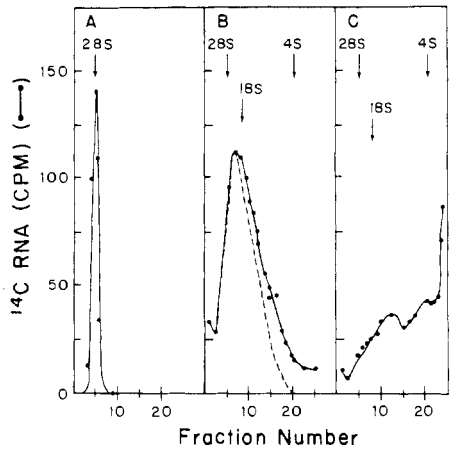

FIGURE 7: Endonucleolytic hydrolysis of RNA by homogeneous *E. coli* polymerase I analyzed by acrylamide gel electrophoresis. (A) 28S [^{14}C]rRNA extracted from L-cells as described in Methods and subjected to a 2-hr electrophoresis at 5 mA/gel (2.7% acrylamide gel, 10 cm × 6 mM) (Perry and Kelley, 1968). The gel is sliced and counted in liquifluor toluene containing 3% protosol. (B) 28S [^{14}C]rRNA extracted from L-cells and incubated in the presence of *E. coli* DNA polymerase I, E/R = 9, for 1 hr in 60 mM Tris-HCl (pH 7) and 8 mM $MgCl_2$ at 37°. The RNA in the reaction mixture was extracted with phenol-chloroform, precipitated with 100 μg of yeast sRNA in 2 volumes of ethanol overnight at −20°. It was then taken up in 30% sucrose buffer (pH 7.2) (120 mM Tris-60 mM NaOAc- and 3 mM EDTA) and applied to a 2.7% acrylamide gel for electrophoresis as described for (A). The dotted line indicates the ideal distribution curve for a 28S molecule which has received two random cuts. The solid line is a plot of the experimental data. (C) The same as (B) but the incubation mixture also contained 100 μM each of dATP, dGTP, dCTP, and dTTP. The direction of RNA migration is from left to right.

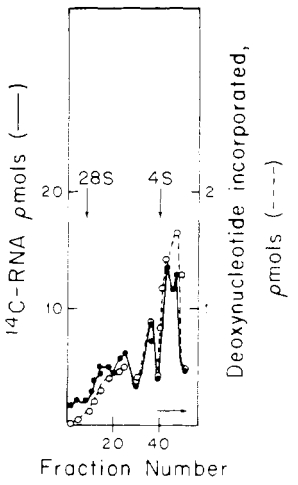

FIGURE 8: Association of DNA product with ^{14}C-labeled L-cell RNA analyzed by acrylamide gel electrophoresis. See legend to Figure 7 for methodology; the reaction mixture is the same as described for Figure 7C and the time of incubation is 30 min.

tions of Mg^{2+}, one can minimize poly[d(A-T)] synthesis. However, a maximal rate of DNA synthesis is achieved at 8 mM Mg^{2+}, at 6 mM Mg^{2+} it is 60%, and at 12 mM Mg^{2+} it is 40% this rate.

The problem of *"de novo"* formation of poly[dG]·poly[dC] which Pol I has been reported to catalyze did not pose a problem in our reactions because a lag period of 40–50 hr is necessary (Burd and Wells, 1970).

Effect of the Nuclease Activities of Pol I on the Polymerization Reaction. The role of the nuclease activities, which are known to be part of Pol I, was investigated. These nucleases might degrade either the template or the product during the reaction. Pol I has been shown to carry out three types of nucleolytic degradation: the 3′- and 5′-DNA exonucleases described by Deutscher and Kornberg (1969a,b), and the 5′ RNase H exonuclease activity reported by Leis *et al.* (1973).

The exonuclease activitis of Pol I were analyzed by measuring the amount of template made acid soluble at various incubation periods. Less than 2% of the template becomes acid soluble in 1 hr, and only 10% of it is solubilized after a 4-hr incubation. When the acid-soluble fraction from a 4-hr reaction was analyzed by paper chromatography, the hydrolysate contained no detectable mononucleotides, although it did contain di-, tri- and larger nucleotide oligomers. This is consistent with the results found by Leis *et al.* (1973) for the RNase H exonuclease activity from *E. coli*.

Pol I appears to have an associated endonuclease activity. When Pol I is incubated for 1 hr at 37° with 28S RNA (E/R = 9) in the presence of Mg^{2+}, the RNA is never found to have more than two cuts. These cuts appear to be random: when we used a statistical method developed by Litwin (1969) for the distribution of randomly cut, completely denatured viral DNA molecules sedimented on sucrose gradients, we were able to plot ideal distribution curves for molecules with variable numbers of cuts in a molecule. The experimental data obtained when the RNA is incubated with the enzyme and Mg^{2+} (Figure 7B) correspond to the ideal distribution curve when the number of cuts in the molecule is two. A similar distribution of molecules is obtained when the ^{14}C-labeled L-cell RNA is preincubated with 70% formamide for 30 min at 35° (Suzuki *et al.*, 1972). The formamide denatures the RNA to form a molecule with little secondary structure. The deviation from the ideal curve may possibly be explained by the fact that the cuts in the 28S RNA are not completely random. When RNA is incubated with the complete reaction mixture under conditions which permit DNA synthesis, the number of RNA fragments is greatly increased, and the cuts in the molecules appear to be discrete (Figure 7C).

The degree of RNA breakdown appears to be a function of enzyme concentration. In an 1-hr incubation at 37° with the complete reaction mixture (E/R = 9, Figure 7C), several classes of molecular lengths are found: approximately 25% of the molecules have fewer than two cuts; 30% of the initial population of molecules has eight cuts; 20%, 69 cuts; and 25%, greater than 400 cuts. If E/R is greater than 11, over 90% of the molecules have more than 100 cuts. However, when E/R is reduced, *i.e.*, when E/R = 3, over 40% of the molecules have less than eight cuts, and at E/R = 0.3, 38% of the molecules have an average of two cuts and 25%, eight cuts. Similar distributions of molecular lengths occur with *Drosophila* 28S RNA.

Since the RNA template is fragmented, it became of interest to learn whether only molecules of certain selected size classes were copied. To do this, L-cell ^{14}C-labeled 28S RNA was used as the template (E/R = 9) and the amount of polymerization was measured by the uptake [^{3}H]dCTP. A gel analysis of the phenol-extracted product of the reaction (Figure 8) shows that in an incubation period as short as 30 min, the DNA product is intimately associated with all sizes of template. Since the template was fragmented during the process of the polymerization reaction and the product appeared to be associated with all size fragments, we were curious to learn how much of the polymerase was actually bound to the template during catalysis. The binding of the enzyme to the template was analyzed by sucrose gradient sedimentation of the reaction mixture followed by assay of each gradient fraction for enzyme activity using activated DNA. We based our experiment on the following assumptions: (1) the enzyme would remain bound to the RNA

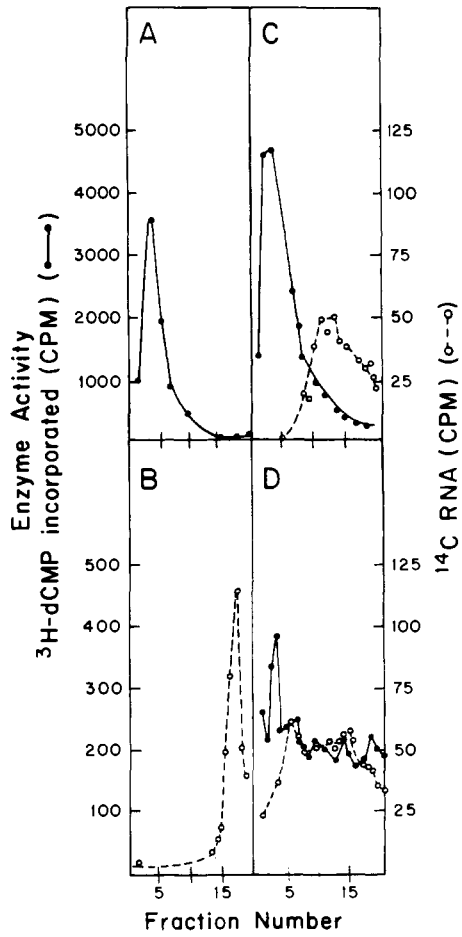

FIGURE 9: Sedimentation of enzyme-template complex in sucrose density gradients. (A) Sedimentation of 15 pmol of Pol I on a 5-25% sucrose gradient containing 0.01 M Tris (pH 7.4)-0.1 M NaCl, centrifuged at 45,000 rpm at 4° for 3.5 hr in a Spinco SW 50.1 rotor; the enzyme activity was assayed as described in Methods when activated DNA is used as a template. L-cell [^{14}C]RNA containing 4S, 18S, and 28S RNA was used as a marker and run on a separate gradient in the same rotor. (B) Sedimentation of 1 pmol of L-cell ^{14}C-labeled 28S RNA on a 5-25% sucrose gradient as described above. The gradient was fractionated from the top and each fraction assayed for radioactivity after the addition of scintillating fluid. (C) A reaction mixture described in Methods for copying RNA with an E/R ratio equal to 10 was immediately applied to a sucrose gradient. The [^{14}C]RNA template and enzyme activity were determined as described above. (D) The same as (C), except that the reaction mixture contained an E/R = 1.

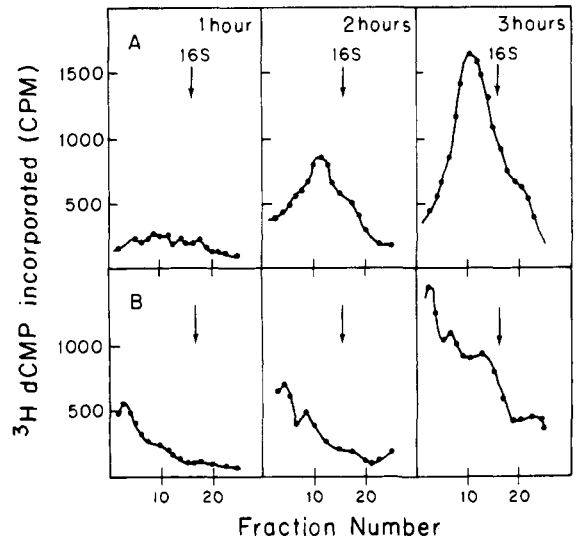

FIGURE 10: Gradient sedimentation analysis of reaction products. The reaction mixture is that described in Methods using an E/R = 3. The incubation times are those stated on the graph. The reaction mixture was diluted to 5 ml with 0.01 M Tris-HCl (pH 7.4)-1 mM EDTA, extracted with phenol-chloroform (Loeb *et al.*, 1973), and centrifuged on a sucrose gradient as described in the legend to Figure 9. (A) Phenol extracted reaction products. (B) The RNA-DNA hybrid. Same as (A), but the single-stranded regions of template RNA were removed by incubating the reaction products with 0.1 vol of RNase A (1 mg/ml, Worthington) for 1 hr at 37° and 0.01 vol of Pronase (5 mg/ml) for 1 hr at 37° prior to phenol extraction.

template on a sucrose gradient containing 0.1 M NaCl [Englund *et al.* (1969) have shown that binding of the enzyme is stable for DNA under these conditions]; (2) the polymerase exchanges the RNA template for an "activated" DNA template when the DNA is present in large excess (1000-fold with respect to 3′-OH termini); and (3) there is little polymerization of DNA or degradation of the RNA template during a 3.5-hr centrifugation at 4°. When the binding of the enzyme to the template is analyzed, as described, several observations are apparent (Figure 9). The enzyme alone has an *s* value of ~5 (Figure 9A); the 28S RNA sediments as shown in Figure 9B. When the enzyme is mixed with the RNA, it binds and migrates with the RNA on a sucrose density gradient (Figure 9C and D). The amount of enzyme migrating with the RNA is the same whether the ratio E/R is 10 or 1, *i.e.*, only 12% of the enzyme activity migrates with the RNA on the sucrose gradient when E/R is 10 (Figure 9C), whereas approximately 80% of the enzyme activity migrates with the template when the E/R ratio is 1 (Figure 9D). Thus, in the absence of measurable polymerization there appears to be a 1:1 stoichiometry between polymerase and template. However, binding of enzyme molecules in excess of a molar ratio of 1 and, as a result, the further fragmentation of the RNA template seem to be dependent on the onset of DNA synthesis (Figure 7).

Analyses of the products of the RNA dependent DNA polymerization reaction are illustrated in Figure 10. In Figure 10A, sedimentation analysis of the phenol-extracted products obtained at three incubation times reflects the molecular size of the RNA template. However, when the uncopied part of the RNA template, *i.e.*, the single-stranded RNA, is hydrolyzed by RNase and the reaction products extracted with phenol, the molecular size of the RNA-DNA hybrid is obtained (Figure 10B). In both analyses, the amount of DNA synthesized from each sized template appears to increase with increased incubation times but it is not obvious that the product elongates extensively over these prolonged periods of incubation.

Discussion

Our experiments suggest that the RNA dependent DNA polymerase activity of Pol I is an integral part of the enzyme. Pol I's RNA dependent activity cannot be separated from its DNA dependent activity either by gel filtration or by isoelectric focusing. The fact that enzyme-bound Zn^+ is necessary for both template activities further substantiates the observation that both functions are carried out by the same enzyme. Springgate *et al.* (1973) have shown that Pol I contains 1 g-atom of Zn/mol of enzyme, and that it is necessary for DNA dependent polymerization. We found that the RNA dependent activity decreases to the same extent as the DNA dependent activity when Zn^{2+} is removed from the enzyme.

We have shown previously (Loeb *et al.*, 1973) that the general requirements for RNA dependent catalysis with Pol I are the same as those for the DNA polymerases from the oncornaviruses ("reverse transcriptases"). The reaction requires a 3′-OH initiator for attachment, Mg^{2+} or Mn^{2+} as a metal activa-

tor, and the complementary deoxynucleoside triphosphates as substrates. Our experiments indicate that the DNA product is covalently attached to the RNA. This suggests that the 28S RNAs contain double-stranded regions which can provide 3′-OH initiator ends, either by phosphodiester breaks in one strand or by a natural "hair pin" loop at the 3′-OH end of the molecule. Gulati *et al.* (1974) using hybridization analyses have shown that the extent to which Pol I will copy a variety of natural RNA molecules is about the same as that for the AMV DNA polymerase. However, the DNA product, using Pol I under their conditions, is smaller (4 S) than that obtained by the AMV polymerase (6–8 S).

The ratio of enzyme to RNA in the reaction mixture appears to be critical for the size of the product. The rate of deoxynucleotide incorporation is constant for several hours except at high concentrations of enzyme when the amount of incorporation decreases upon extended incubation. If large amounts of DNA product are desired, an E/R ratio = 10 is recommended for a 3-hr incubation period at 37°.

Long incubation periods are complicated, however, by the formation of *"de novo"* d(A-T) by Pol I. We have shown that the concentration of Mg^{2+} is a critical factor in determining the occurrence of poly[d(A-T)] synthesis. At 4 mM Mg^{2+} the lag in the synthesis of d(A-T) is less than 1 hr. Above 4 mM Mg^{2+} the lag period can be increased to a maximum of about 3 hr. However, since the rate of nucleotide incorporation into product decreases at about this time due to other complications, it is not expedient to prolong the incubation period beyond 3 hr.

Pol I has been shown to have associated nucleases, 3′ and 5′ exonucleases, and an RNase H 5′ → 3′ exonuclease activity by others. We found that Pol I also has what appears to be an RNA endonuclease which is activated by the conditions of DNA synthesis. When a 28S RNA template is incubated for 1 hr with only Pol I (E/R = 9) and Mg^{2+}, each 28S molecule has approximately two endonucleolytic breaks. Denaturation of the 28S RNA with 70% formamide indicates that these breaks may have been already present as single nicks in the double-stranded parts of the 28S RNA molecule prior to its incubation with Pol I and Mg^{2+}. Upon the addition of the four deoxynucleoside triphosphates to the above mixture, the RNA template is cut into several classes of discrete molecular lengths. The fact that the endonucleolytic reaction takes place concurrently with the polymerization reaction, and that the magnitude of both reactions is a function of enzyme concentration, argues that Pol I can act as an endonuclease to hydrolyze RNA. The discreteness of molecular size classes of the hydrolyzed RNA at low enzyme concentrations may be a consequence of the requirements of the endonuclease for specific activation sites and/or the secondary structure of the RNA.

The endoribonuclease activity which we have observed associated with Pol I, and which can be measured before any amount of DNA synthesis occurs, does not appear to be the same as that associated with the AMV DNA polymerase described by Baltimore and Smoler (1972). This AMV endoribonuclease activity requires Mg^{2+}, produces 3′-OH ends on the RNA, is synthesis dependent, and requires an RNA–DNA hybrid as template.

An RNase H endonuclease which is separate from Pol I has been isolated from *E. coli* and described by Berkower *et al.* (1973). This endonuclease can be inhibited by *N*-ethylmaleimide, whereas the endonuclease activity of Pol I cannot. Another enzyme which has been isolated from *E. coli*, RNase III (Robertson *et al.*, 1968) appears to have an endonuclease activity which requires substrates containing double-stranded polyribonucleotide regions and Mg^{2+}; however, it produces 5′-OH ends on the RNA unlike Pol I which produces 3′-OH ends.

Leis *et al.* (1973) have reported a 5′ → 3′ RNase H exonuclease activity for intact Pol I although Henry *et al.* (1973) have not been able to show this activity in the small fragment of Pol I, *i.e.*, that fragment which contains the 5′ → 3′ DNA exonuclease activity. Our results show an RNase H exonuclease comparable to that of Leis *et al.* (1973) present in the intact polymerase. During DNA synthesis, however, this activity is very low, 7% of the RNA template is hydrolyzed to acid-soluble products at 37° in 3 hr (E/R = 9). This apparent low activity may be a measure of the fact that only 7% of the RNA template is in hybrid form with available 5′-OH termini.

Our results show that some DNA molecules larger than 16 S can be synthesized from 28S RNA templates and that the amount of large molecules synthesized can be controlled by changing the ratio of Pol I to template in the reaction mixture. Molecules in the range of 6–9 S have been synthesized by the oncorna virus polymerases using a 10S RNA template. However, most DNAs synthesized with different RNA templates, and viral RNA dependent DNA polymerases, have been smaller in size (for a discussion, see Taylor *et al.*, 1973). Such small molecules would result if the viral RNA dependent DNA polymerases contained an RNA endonuclease activity similar to the one described here for Pol I.

The initiation of DNA synthesis by Pol I on RNA may be determined by the specificity of its RNA endonuclease. Most RNases which produce a 3′-OH end prefer adenine as the base next to the 3′ diester bond, and only when the RNA chain becomes shortened does this specificity disappear (Barnard, 1969). This type of specificity agrees with Faras *et al.*'s (1973) observation that the RNA directed DNA polymerase of *Rous sarcoma* virus with 70S viral RNA template initiates by the covalent attachment of dAMP to the 3′-terminal adenosine of an RNA molecule.

The initial nicking of the RNA template appears to happen very quickly and prior to or concurrent with the onset of polymerization. The product appears to be associated with all size RNA molecules in the reaction mixture even during short incubation periods. Also, there seem to be a finite number of sites at which Pol I initially binds to the RNA template whether the E/R ratio is 10 or 1. When the product of the reaction is analyzed at various times of incubation at a given E/R ratio, primarily the amount, not the size, of DNA synthesized from each template appears to increase with increased incubation times.

These results would explain the apparent paradox which has existed in studies of the RNA dependent DNA synthetic reaction, *i.e.*, that although the final DNA product of such a reaction is small compared to the size of the template, most of the sequences of any natural RNA template are faithfully copied (Temin and Baltimore, 1972; Garapin *et al.*, 1973; Taylor *et al.*, 1973; Gulati *et al.*, 1974) as determined by molecular hybridization experiments.

Our observations led to a tentative model of how Pol I copies RNA (Figure 11). Ideally, Pol I would only attach to the single 3′-OH initiator end of a 28S RNA molecule which might exist, because of a "hair pin" loop region, at one end of the template. Once the enzyme is bound, it would proceed to copy the remainder of the RNA template. Possibly, this does happen with certain templates: (1) if the RNA template is relatively free of secondary structure, and (2) if every Pol I molecule is bound to the 3′-OH end of an RNA molecule. However, 28S ribosomal RNA is not free of secondary structure: the 28S rRNA molecules from HeLa cells when spread and examined by the electron microscope have a highly reproducible secondary structure

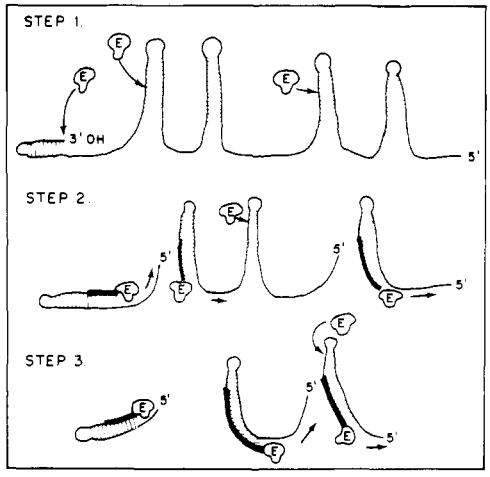

FIGURE 11: Model proposed for the initiation of DNA synthesis with an RNA template using Pol I. Step 1: Diagrammatic representation of 28S rRNA having double-stranded regions and a 3′-OH looped terminus. Step 2: The endonuclease associated with Pol I attacks the double-stranded regions of 28S RNA to produce more 3′-OH ends for the initiation of DNA synthesis by the polymerase. Step 3: Continuation of DNA synthesis and production of new 3′-OH termini.

of double-stranded "hair pin" loops at definite intervals along their length (Wellauer and David, 1973). It is interesting to note that these investigators find little secondary structure in 18S rRNA. Our own experiments corroborate this: oligonucleotides have to be added to 18S RNA to furnish initiation sites for Pol I. However, when secondary structure is present in the RNA, *i.e.*, 28S RNA, Pol I appears to create its own 3′-OH initiation sites. Our evidence suggests but does not prove that *E. coli* Pol I has an endonuclease activity which nicks a single strand of the double-stranded part of the RNA template to create a 3′-OH end and then proceeds to copy that moiety of the RNA template which remains before it. Except for the requirement of double strandedness, these nicks are most likely random. Once the polymerase is covalently bound to the template, it no longer appears to be able to cut it, but proceeds to copy it. If, indeed, this is the model reaction, it should be possible to copy entire RNA templates which have a small degree of secondary structure with relatively low concentrations of Pol I without cutting the template, and then, with increased incubation times and more enzyme, to produce many DNA copies.

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