DNA DEPENDENT RNA POLYMERASE CATALYZED SYNTHESIS OF POLYRIBONUCLEOTIDE CHAINS COVALENTLY LINKED TO DNA

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SUMMARY: RNA polymerase of Escherichia coli catalyzes the covalent attachment of ribonucleotides to 3'-OH ends of DNA chains. This was verified by (a) the isolation of hybrid structures in CsCl gradients containing RNA and DNA after denaturation in the presence of HCHO and (b) the isolation of DNA containing ribonucleotides after alkaline hydrolysis.

INTRODUCTION

RNA polymerase is capable of initiating new RNA chains (1,2) and adding to the 3'-OH ends of pre-existing RNA chains (3,4). However, when RNA polymerase products made with dAT copolymer were banded in CsCl or Cs_2SO_4 density gradients, 50 to 90% of the product was detected at the density of DNA-RNA hybrids before and after denaturation in the presence of formaldehyde. This, and other findings presented below, indicate that in addition to the two reactions described above RNA polymerase can catalyze the covalent attachment of ribonucleotides to 3'-OH ends of DNA chains.

MATERIALS AND METHODS

All reagents and enzymes used were as previously described (5,6). The dAT copolymer utilized in experiments described below was nicked with pancreatic DNase and possessed an average chain length of 600 nucleotides as measured with 5'-hydroxyl polynucleotide kinase and γ-^{32}P ATP after alkaline phosphatase treatment. E. coli RNA polymerase and core RNA polymerase were as described in the accompanying paper.

Physical Characterization of Products Formed with dAT copolymer. Labeled RNA polymerase products formed with dAT copolymer were banded in HCHO-CsCl gradients after denaturation in the presence of HCHO (Fig 1). Free ^{32}P-RNA, as expected, was detected near the bottom of the tube in the region of high density (ρ = 1.780 g/cc). Substantial ^{32}P-RNA product was detected in the region characteristic of RNA-DNA hybrids, suggesting that the ^{32}P-RNA material banding at

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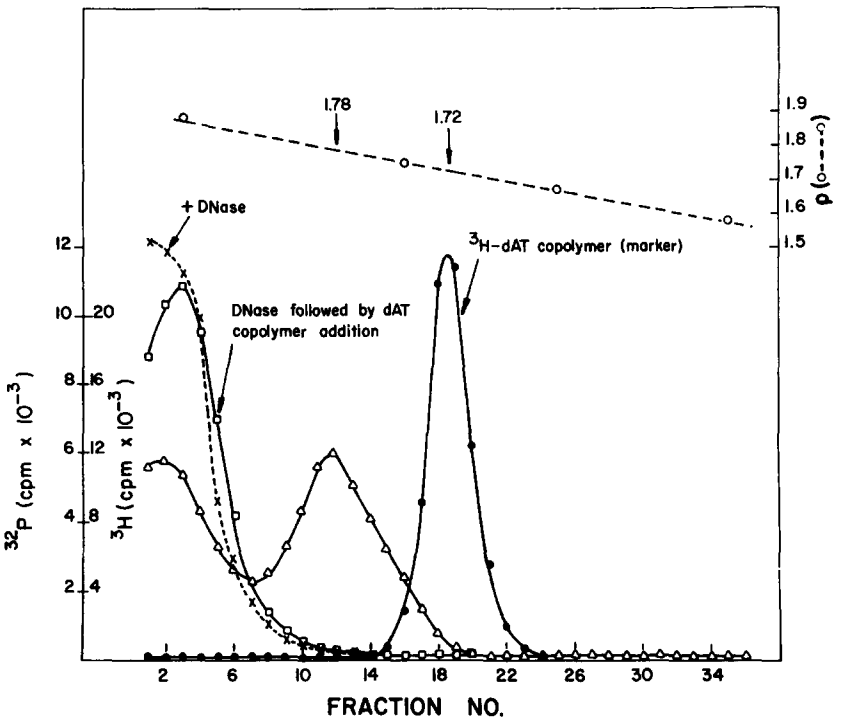

Figure 1. HCHO-CsCl Gradient Centrifugation of Poly rAU Product. A reaction mixture (0.63 ml) containing 7.5 nmoles of α^{32}P-UTP (64 cpm/pmole), 30 nmoles ATP, 30 μmoles of Tris buffer, pH 7.9, 6 μmoles of $MgCl_2$, 60 nmoles of nicked dAT copolymer (average chain length of 600 nucleotides), 3 μg bovine serum albumin, 0.3 μmole of dithioerythritol and 15 units of RNA polymerase was incubated for 20 min at 38°. The polymerization reaction was halted by heating the mixture for 5 min at 65°. The mixture was divided into 3 aliquots of 0.2 ml each. Two of these aliquots were treated with 10 μg of pancreatic DNase and all aliquots were incubated for 30 min at 38°. Each reaction mixture then received 0.02 ml of 1 M EDTA, 0.035 ml of 1 M sodium phosphate buffer, pH 7.0, 0.005 ml of 10% Sarkosyl, 0.15 ml of 10 M HCHO, 1 nmole of ^{3}H-dAT (as marker) and 1 ml of H_2O. One of the DNase treated aliquots also received 20 nmoles of dAT copolymer. The mixtures were then heated at 85° for 10 min, cooled, and diluted to a volume of 3 ml. Solid CsCl (3.7 gms) was added to each and the mixtures were centrifuged at 45,000 rpm in an SW50 rotor at 30° for 40 hours. After centrifugation fractions were collected, refractive indices measured, and acid-insoluble radioactivity determined.

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this density was attached to DNA. As shown in Fig 1, the denaturing conditions employed prevented dAT copolymer from hydrogen-bonding to the poly rAU product; the DNase-treated product (after heat-inactivation of the DNase) banded at the density of RNA when fresh dAT copolymer was added. Identical experiments were carried out with Cs_2SO_4 in place of CsCl and the results were the same; 50% of the product banded at a hybrid density (ρ = 1.52 g/cc). Thus the material

TABLE 1

RIBONUCLEOTIDE ADDITION AT ENDS OF DNA CHAINS

ADDITIONS		ACID-INSOLUBLE ^{32}P (pmoles)	
Enzyme	Nucleotides	Before Alkaline Hydrolysis	After Alkaline Hydrolysis
Holoenzyme	α^{32}P-UTP + ATP	750	3.70
	α^{32}P-ATP + UTP	760	4.51
Core	α^{32}P-UTP + ATP	470	1.81
	α^{32}P-ATP + UTP	480	1.97

Reaction mixtures (0.1 ml) containing either 1.25 nmoles of α^{32}P-ATP (3000 cpm/pmole) and 5 nmoles of UTP or 1.25 nmoles of α^{32}P-UTP (2500 cpm/pmole) and 5 nmoles of ATP, 9.8 nmoles of dAT copolymer (with an average chain length of 600) 0.05 μmole of dithioerythritol, 1 μmole of $MgCl_2$, 0.5 μg bovine serum albumin and 2 units of RNA polymerase were incubated for 20 min at 38°. Reactions were halted with 0.1 ml 0.1 M sodium pyrophosphate, 0.005 ml 10 mg/ml albumin and 3 ml of 5% trichloroacetic acid. The insoluble material was collected by centrifugation and dissolved in 0.5 ml of 1 N NH_4OH; the solution was acidified with 3 ml of 5% TCA and the procedure repeated two more times. The final pellet was dissolved in 0.75 ml of 0.5 N NaOH and incubated for 18 hrs at 38°. Aliquots were taken for acid-insoluble ^{32}P determination prior to the acid-precipitation as well as after alkaline hydrolysis.

banding at the hybrid density most likely represents a covalent association between dAT copolymer and poly rAU.

Chemical Characterization of Products. Further evidence of the covalent attachment between incorporated ribonucleotides and DNA primer was obtained by studying the amount of labeled ribonucleotide remaining acid-insoluble after alkaline hydrolysis (Table 1). Approximately 0.4 to 0.5% of incorporated ribonucleotides remained acid-insoluble after alkaline hydrolysis; this fraction was not altered if the core RNA polymerase was utilized in place of the holoenzyme. The acid-insoluble material (formed with core or holoenzyme) detected after alkaline hydrolysis was subjected to alkaline-CsCl density gradient centrifugation. All the ^{32}P-banded at the density expected for dAT copolymer (Fig 2).

Properties of End Group Addition. The above experiments indicate covalent attachment of ribonucleotides to DNA. Covalent attachment of ribonucleotides to DNA was more conveniently studied using a single ribonucleoside triphosphate.

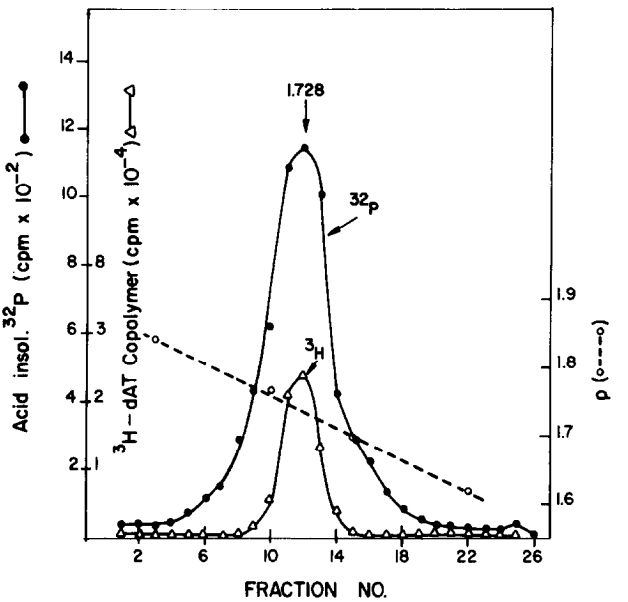

Figure 2. Alkaline CsCl Gradient Centrifugation of Alkali-Stable Product. The alkaline hydrolyzed products were prepared as described in the legend to Table 1, with holoenzyme. A portion of this material (0.45 ml containing 6200 cpm) was treated with 0.02 ml of 1 M EDTA, 1 nmole of ^{3}H-dAT copolymer marker, 1.73 ml of 0.25 N NaOH and 2.73 gm of solid CsCl and the reaction mixtures were centrifuged at 45,000 rpm in an SW50 rotor for 40 hours at 25°. All measurements were as described in Fig 1. The results presented above were obtained with $\alpha^{32}P$-UTP. Identical results were observed with products labeled with $\alpha^{32}P$-ATP. The recovery of acid-insoluble ^{32}P after centrifugation was 94% in the above experiment. Similar results have been obtained with core enzyme and the above ribonucleotides.

To avoid possible complications due to trace amounts of contaminating ribonucleotide all products were subjected to alkaline hydrolysis before acid precipitation. Employing such conditions, the requirements of the reaction with dAT copolymer as primer were examined (Table 2). The incorporation of either AMP or UMP was dependent on dAT copolymer; poly dI:dC homopolymer could not substitute for dAT copolymer. The addition of DNase eliminated the reaction while RNase had no effect. The reaction was reduced by the addition of relatively large amounts of rifampicin and streptolydigin, while the simultaneous addition of both inhibitors quantitative abolished nucleotide incorporation. It was observed, as shown in Table 2, that UMP incorporation exceeded AMP incorporation. The addition of ribonucleotides to ends of DNA chains was not stimulated by Mn^{++} and for this reason, Mg^{++} was used in all experiments.

Further proof that ribonucleotides were incorporated into DNA was obtained by the isolation of the labeled product following alkaline hydrolysis.

TABLE 2

INCORPORATION OF SINGLE RIBONUCLEOTIDE

	ADDITIONS	INCORPORATION OF AMP or UMP (pmoles/15 min)	
1.	Complete	2.0	4.60
2.	" + Rifampicin (25 μg/ml)	0.87	1.07
3.	" + Streptolygidin (5.5×10^{-4} M)	0.42	1.11
4.	" + Rifampicin + streptolydigin	<0.03	0.07
5.	" omit dAT copolymer	<0.03	<0.03
6.	" with dI·dC in place of dAT copolymer	<0.03	<0.03
7.	" + DNase (1 μg)	<0.03	<0.03
8.	" + RNase (2.5 μg)	1.88	4.51

Reaction mixtures (0.1 ml) contained: 1.25 nmoles of α^{32}P-ATP (1300 cpm/pmole) or 1.25 nmoles of α^{32}P-UTP (2300 cpm/pmole), 1 μmole of $MgCl_2$, 5 μmoles of Tris buffer, pH 7.9, 5 nmoles of dAT copolymer, 0.05 μmole of dithiothreitol, 0.5 μg bovine serum albumin, and 2.4 μg of RNA polymerase. After incubation at 38°, reactions were halted with 0.2 ml of 1 N NaOH, heated at 100° for 10 min, cooled and 0.1 ml of 0.1 M sodium pyrophosphate, 125 nmoles of salmon sperm DNA and 3 ml of 10% trichloroacetic acid were added. The mixtures were filtered through glass fiber millipores (GF/C). In experiments where rifampicin and streptolydigin were combined the amounts indicated individually were added. The amount of dI·dC utilized in place of dAT copolymer was 5 nmoles.

Polymerase products formed with dAT copolymer and α^{32}P-UTP were degraded to 5'-mononucleotides with snake venom phosphodiesterase and pancreatic DNase. After electrophoresis, all incorporated ^{32}P was recovered in the UMP (TMP) region. The 5' mononucleotide product (18,000 cpm) was subjected to chromatography in isopropanol-NH_3-borate (7) to separate 5'-UMP and 5'-TMP. More than 99% of the ^{32}P was recovered with the 5'-UMP marker. Thus, all the ^{32}P incorporated into an acid-insoluble form with UMP was indeed ribonucleotides covalently incorporated into DNA. Similar experiments were carried out with identical results with α^{32}P-ATP

The kinetics of the incorporation of UMP or AMP were examined (Fig 3 A and B). As shown, incorporation of AMP reached a plateau at 2 min and further incubation resulted in only a slight increase in incorporation under the conditions employed; in contrast UMP incorporation continued for a prolonged period. This

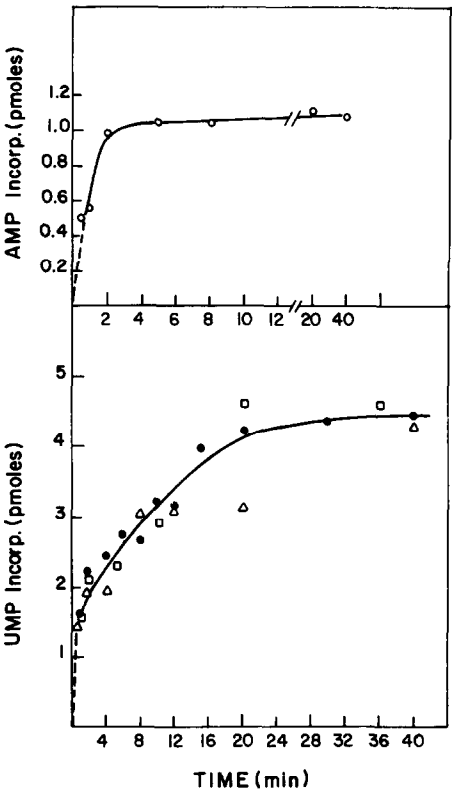

Fig. 3.

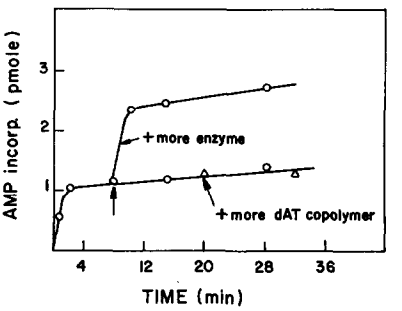

Fig. 4.

Figure 3. Kinetics of End Group Addition. Additions and conditions used were as described in Table 2 with 4.2 units (2.1 μg) of RNA polymerase and 12.8 nmoles of dAT copolymer. Reactions were halted by the addition of 0.1 ml 1 N NaOH followed by alkaline hydrolysis. In Fig 3A, AMP incorporation was followed while in Fig 3B UMP incorporation was measured. In the latter case 3 different experiments are plotted. Each experiment is indicated by a different symbol.

Figure 4. Influence of Various Additions on Extent of AMP Incorporation into DNA Chains. Reaction mixtures were as described in Table 2 with 2.1 μg of RNA polymerase and 12.8 nmoles of dAT copolymer. After 8 min at 38° a series of reaction mixtures received an additional 12.8 nmoles of dAT copolymer (Δ————Δ), while another series received an additional 2.1 μg of RNA polymerase (0————0). Reactions were continued for indicated times and stopped by the addition of 0.1 ml 1 N NaOH followed by alkaline hydrolysis at 100° as in Table 2.

observation may explain the discrepancy in incorporation of these nucleotides noted in Table 2. The continued fixation of UMP as a function of time may represent the recycling of enzyme during the course of the reaction. Similar results were obtained with core RNA polymerase preparations. As shown in Fig 4, once AMP incorporation ceased, addition of more dAT copolymer was without effect

TABLE 3

RELATIONSHIP BETWEEN RNA POLYMERASE, TRIPHOSPHATE TERMINI, AND INCORPORATION OF AMP INTO dAT COPOLYMER

DNA ADDED	RNA POLYMERASE	RIFAMPICIN ADDED	γ^{32}P-NTP INCORPORATED			^{32}P-AMP INCORPORATED with dAT copolymer
			γ^{32}P-ATP	γ^{32}P-GTP	TOTAL	
	μg	μg/ml	pmoles			pmoles
Colicin E_I	3.0	none	1.01	0.97	1.98	2.06
	3.0	1.2	1.05	0.86	1.91	----
	1.5	none	0.43	0.46	0.89	1.04
T4	3.0	none	1.07	0.29	1.36	2.06
T7	3.0	none	1.04	0.49	1.53	2.06

For the determination of triphosphate termini, reaction mixtures (0.1 ml) contained: 5 μmoles of Tris buffer, pH 7.9; 1 μmole of $MgCl_2$; 0.05 μmole of dithiothreitol; 0.5 μg bovine serum albumin; 25 nmoles of CTP; 25 nmoles of ^{3}H-UTP (10,000 cpm/nmole); 15 nmoles of γ^{32}P-GTP (1400 cpm/pmole) and 25 nmoles of ATP, or 15 nmoles of γ^{32}P-ATP (1100 cpm/pmole) and 25 nmoles of GTP; 12 nmoles of colicin E_I DNA, 19 nmoles of T4 DNA, or 29 nmoles of T7 DNA and RNA polymerase as indicated. Reactions were incubated at 37° for 1, 3, 5, 10, 20 or 30 min with or without the addition of 0.125 μg of rifampicin at 2 min. All reactions were halted by addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.5 mg bovine serum albumin, and 3 ml of 5% TCA. Insoluble material was collected by centrifugation, dissolved in 0.2 ml of 0.5 M NaOH at 0° and then acidified with 3 ml of 5% TCA; the procedure was repeated 2 more times. The mixtures were finally filtered through glass fiber millipores (GF/C). Reactions to determine incorporation of AMP into alkali resistant TCA insoluble material with dAT copolymer were as described in the legend to Table 2.

The results presented above for triphosphate termini were an average of results observed after 3 min of synthesis. There was no significant change in the number of triphosphate termini detected after rifampicin addition or after 3 min of incubation.

However, the addition of more enzyme at this point caused an immediate resumption of AMP incorporation and the yield achieved was identical to that obtained after the first stage of AMP incorporation. The cessation of AMP incorporation was not due to inactivation of the enzyme since the addition of UTP at any time resulted in an immediate marked incorporation of AMP.

The covalent linkage of ribonucleotides to 3'-OH ends of DNA chains was not limited to dAT copolymer. Reactions utilizing native T7 or T4 DNA as primer supported the incorporation of labeled ribonucleotides in an alkaline stable acid-insoluble form; in contrast, with colicin E_I DNA, a circular duplex DNA, or heat denatured T4 or T7 DNA, no detectable incorporation was observed.

Quantitative Relationship between Enzyme Addition Covalent Attachment of AMP to dAT and Triphosphate Terminated RNA. At present the number of active RNA polymerase molecules can be measured by determining the quantitative relationship between moles of enzyme added and moles of RNA chains produced in the absence of reinitiation (8). This was done using colicin E_I supercoiled DNA as template and measuring γ^{32}P-ATP and γ^{32}P-GTP incorporation in low salt with or without the addition of rifampicin after 2 min of incubation to prevent reinitiation (Table 3). Approximately 2 pmoles of triphosphate terminated RNA chains were produced by 3 μg (6 pmoles) of RNA polymerase. The same amount of enzyme catalyzed the covalent incorporation of 2 pmoles of α^{32}P ATP to dAT copolymer in the absence of UTP. These values were directly proportional to enzyme concentration; 1.5 μg of protein catalyzed half the incorporation noted with 3 μg of protein. Thus, this preparation of RNA polymerase contained 30-40% active polymerase molecules based on the Bucher (9) and Lowry et al. (10) methods for protein determinations with bovine serum albumin as the protein standard. Since end group addition is independent of the sigma subunit, the discrepancy between the number of RNA polymerase molecules added and the number of active molecules cannot be due to a deficiency in this component.

With linear DNA as primer (such as T4 and T7) significantly lower amounts of triphosphate termini were formed compared with colicin E_I DNA (Table 3). These discrepancies were shown to be due to covalent linkage of ribonucleotides to ends in the DNA chains. These DNA preparations most likely contained a number of single strand breaks.

DISCUSSION

The results presented above indicate that RNA polymerase can catalyze the incorporation of ribonucleotides at ends of DNA chains. Thus, RNA polymerase can catalyze three discrete types of reactions in forming RNA chains: (a) the polymerase can initiate RNA chains de novo (generating triphosphate termini); (b) it can add to 3'-OH ends of RNA chains; (c) it can add to 3'-OH ends of DNA chains.

The finding that the incorporation of AMP at 3'-OH ends of dAT copolymer leads to a more stable end point than with UMP incorporation is similar to the results observed by Helm and Krakow (11). These workers noted a greater stability of the enzyme-dAT copolymer-ATP-streptolydigin complex in comparison to the complex containing UTP in place of ATP.

The end group addition noted here with dAT copolymer is not limited to this DNA but was also observed with a variety of other DNA preparations; however, the reaction was not detected with supercoiled colicin E_I DNA or denatured DNA. There are indications that single strand breaks in DNA inhibit the

RNA polymerase holoenzyme and Hinkle et al. (12) have suggested that RNA polymerase can be sequestered at these breaks. It may be that the polymerase acts at these sites to incorporate nucleotides attached covalently to the template. If so the facility with which RNA polymerases can displace strands will govern whether these single strand breaks become sites of extensive RNA synthesis. If RNA polymerase can incorporate and extend chains from 3'-OH ends of single strand breaks in duplex DNA, the formation of RNA-DNA covalent hybrids becomes an important reaction to consider. In addition it is also evident that the stimulatory effects of single strand breaks in duplex DNA for core polymerase activity (13) may in part be explained by the end group addition described here.

The results presented in Table 3, indicate that the incorporation of ATP into dAT copolymer may be a useful means for determining active RNA polymerase molecules. The fact that this reaction occurs with core as well as holoenzyme permits an accurate estimate of the number of active enzyme molecules, of either form of the enzyme.

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