UTILIZATION OF DEOXYNUCLEOSIDE TRIPHOSPHATES BY DNA-DEPENDENT RNA POLYMERASE OF E. coli*

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SUMMARY. The DNA-dependent RNA polymerase of Escherichia coli catalyzes the concomitant incorporation of ribonucleotides and deoxynucleotides into polymers yielding covalently linked ribo and deoxynucleotides. This reaction is dependent upon the addition of Mn^{++} and is sensitive to rifampicin and streptolydigin.

INTRODUCTION

The enzymatic formation of polynucleotides containing covalently linked ribonucleotides and deoxynucleotides (1-3) has been reported by a number of laboratories. In the past such anomolous syntheses have been considered artifacts of in vitro enzymatic reactions.Recently, two other distinct systems have been shown to involve such covalent structures. An important role for transcription in DNA replication, possibly providing a primer for synthesis, has been reported in a number of biological systems (4-7) and highly purified RNA-dependent DNA polymerase of RNA tumor viruses catalyzes the covalent attachment of deoxynucleotides to 3'-OH ends of RNA primers (8,9). In view of these observations, we decided to reexamine the incorporation of ribonucleotides into DNA-polymers catalyzed by extracts of Escherichia coli. The results presented below indicate that DNA-dependent RNA polymerase of E. coli readily catalyzes the co-incorporation of deoxynucleotides and ribonucleotides into polymers. Such observations have been briefly reported by Cheng and Tso (10) and by Chamberlin (11). In the accompanying paper (12), evidence is presented for the covalent attachment of ribonucleotides to DNA catalyzed by DNA-dependent RNA polymerase.

MATERIALS AND METHODS

E. coli strain D110 (Pol A1, end$^-$) was obtained from Dr. R. Moses and grown as previously described (13). All polymers were obtained as previously described (13) while homogeneous DNA-dependent RNA polymerase of E. coli was prepared by adsorption to DNA cellulose followed by chromatography at low and high salt concentrations on

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agarose columns. The final preparation was freed of contaminating RNase, DNase, and ATPase by glycerol gradient centrifugation and will be detailed elsewhere. The results presented below have been observed with a variety of RNA polymerase preparations prepared by a number of different procedures. The holoenzyme was chromatographed on phosphocellulose as described by Berg et al (14) for the isolation of sigma-deficient core RNA polymerase. The activity of the core preparation was measured with dAT copolymer as primer and was shown to be poorly active with T4 DNA as primer. Antibodies to DNA polymerase I and DNA-dependent RNA polymerase were generous gifts of Drs. I. R. Lehman and U. Maitra, respectively.

RESULTS

Incorporation of ribonucleotides into DNA. Studies with extracts of E. coli strain D110 (end-, pol A1) indicated that labeled ribonucleotides in the presence of deoxynucleotides were incorporated into polydeoxynucleotide chains as detected in previous studies (1). This observation suggested that DNA polymerase I was not the only polymerase capable of forming mixed polynucleotide chains. As shown in Table I. the incorporation of UMP was dependent on added DNA, insensitive to anti-DNA polymerase I stimulated by $MnCl_2$, completely sensitive to DNase but only partly sensitive to pancreatic RNase at the concentrations employed.

The activity responsible for the incoporation of UMP and dAMP in the presence of dAT copolymer was purified approximately 150-fold by chromatography on DEAE cellulose and phosphocellulose. In both cases DNA-dependent RNA polymerase, as measured by UMP and AMP incorporation directed by dAT copolymer, coincided with the incorporating activity observed with dATP and UTP. The rate of heat inactivation of each activity in such purified fractions was identical and both activities were inhibited by antibody preparations directed against highly purified E. coli DNA-dependent RNA polymerase (Table 2). Further indications that RNA polymerase was responsible for the observed incorporation was obtained. In these experiments both ribo- and deoxynucleotide incorporation were inhibited by rifampicin and streptolydigin, two specific inhibitors of transcription (Table 3). In addition, as observed with crude enzyme fractions, deoxynucleotide incorporation catalyzed by purified RNA polymerase was markedly stimulated by Mn^{++}. With dAT copolymer as primer the reaction was dependent on both ATP and dTTP. In all subsequent experiments highly purified homogeneous RNA polymerase was employed.

The sigma deficient form of RNA polymerase (core) and the holoenzyme were examined further for their ability to support deoxynucleotide incorporation (Table 4). Both preparations catalyzed deoxynucleotide incorporation though quantitative differences between the two forms were noted. In experiments not reported here, it was observed that holoenzyme preparations were stimulated by Mn^{++} to a greater extent than core preparations. As shown (Table 4), dTMP incorporation in the presence

TABLE 1

INFLUENCE OF DEOXYNUCLEOTIDES ON RIBONUCLEOTIDE INCORPORATION

Additions	DNA Added	Incorporation pmoles/20 min
1. α^{32}P-UTP + dATP + dGTP + dCTP	thymus	21.3
2. as in 1	none	0.8
3. α^{32}P-UTP + dATP	dAT copolymer	48.8
4. as in 3 + anti-DNA polymerase I	"	50.5
5. 3 - $MnCl_2$	"	6.3
6. 3 + RNase (5 μg)	"	9.8
7. 3 + DNase (5 μg)	"	<0.2

Reaction mixtures (0.1 ml) contained 3.4 nmoles α^{32}P-UTP (1000 cpm/pmole), 5 nmoles each of dATP, dGTP and dCTP, 1 μmole of $MgCl_2$, 0.3 μmole of $MnCl_2$, 5 μmoles of Tris buffer, pH 7.9, 0.2 μmole of dithiothreitol, 20 nmoles of calf thymus DNA, 0.05 μmole of phosphoenolpyruvate, 0.2 μg of phosphopyruvate kinase and 32 μg of E. coli D110 extract treated with polyethylene glycol-dextran followed by dialysis (10). With dAT copolymer as primer, reaction mixtures contained α^{32}P-UTP, 5 nmoles of dATP and 5 nmoles of dAT copolymer in place of thymus DNA. After 20 min at 38° reactions were treated with 0.1 ml 0.1 M sodium pyrophosphate, 100 nmoles of salmon sperm DNA (as carrier) and 5% TCA. Acid-insoluble material was collected on GF/C glass fiber discs, dried and counted in a scintillation counter as previously described (10).

of ATP occurred at approximately 5% of the rate of AMP incorporation in the presence of UTP. When deoxynucleotides were the sole substrates employed, the reaction rate was reduced to 0.5% of the rate of RNA synthesis with ribonucleoside triphosphates. In the latter case, approximately 50% of incorporated deoxynucleotide remained acid-insoluble after alkaline hydrolysis.

Chemical Characterization of the Product. The products expected in reactions primed with dAT copolymer formed with dATP + UTP and ATP + dTTP are the repeated sequences $(dApU)_n$ and $(dTpA)_n$. In either case, ^{32}P introduced into the mixed polymer with α^{32}P-labeled ribonucleoside triphosphates should yield dinucleotides after alkaline hydrolysis in which the ^{32}P is internucleotide (dA$\overset{*}{p}$Up or dT$\overset{*}{p}$Ap). The ^{32}P present in these dinucleotide products should be resistant to the action of phosphomonoesterase but completely susceptible after

TABLE 2

INFLUENCE OF ANTI-RNA POLYMERASE ON DEOXYNUCLEOTIDE DEPENDENT RIBONUCLEOTIDE INCORPORATION.

Addition	^{3}H-AMP Incorporation pmoles/20 min
1. UTP + ^{3}H-ATP	67
2. 1 + anti-RNA polymerase	1.8
3. dTTP + ^{3}H-ATP	6.3
4. 3 + anti-RNA polymerase	0.9

The additions were made in the following order: reaction mixtures (0.09 ml) containing 5 nmoles of dTTP or 4 nmoles of UTP; 1 μmole of $MgCl_2$, 0.15 μmole of $MnCl_2$, 5 μmoles of Tris buffer, pH 7.9, 0.18 unit of RNA polymerase, 0.5 μg of albumin and 0.001 ml of anti-RNA polymerase were incubated for 5 min at room temperature. Nucleotide incorporation was initiated by the addition of 5 nmoles of dAT copolymer and 1.5 nmoles of ^{3}H-ATP (308 cpm/pmole). All other conditions were as described in Table 1. In experiments not shown, DNA polymerase I was unaffected by anti-RNA polymerase and anti-DNA polymerase I had no effect on the RNA polymerase system.

TABLE 3

INFLUENCE OF VARIOUS ADDITIONS ON INCORPORATION

Additions	Incorporation of ^{32}P-AMP	Incorporation of ^{3}H-dTMP
	pmoles/20 min	
1. Complete	43	47.5
2. complete + rifampicin (25 μg/ml)	4.0	2.2
3. complete + streptolydigin (5.5×10^{-4} M)	0.1	0.8
4. complete - omit Mn^{++}	3.9	1.4
5. complete - omit ATP	----	1.4
6. complete - omit TTP	0.68	----

Reaction mixtures (0.1 ml) contained 1.5 nmoles α^{32}P-ATP (200 cpm/pmole) 1 nmole of ^{3}H-dTTP (870 cpm/pmole), 5 μmoles of Tris buffer, pH 7.9 1 μmole of $MgCl_2$, 0.15 μmole of $MnCl_2$, 5 nmoles of dAT copolymer, 0.5 μg of albumin, 1.08 unit of RNA polymerase and inhibitors as indicated.

TABLE 4

INCORPORATION OF NUCLEOTIDES

Enzyme Fraction	^{3}H-TTP + ^{32}P-ATP		^{32}P-ATP + UTP	^{3}H-dTTP + dATP
	pmoles/20 min			
Holoenzyme	42	50	825	4.3
Core	16.2	15	720	3.3

Reaction mixtures (0.1 ml) contained either 1.5 nmoles of ^{32}P-ATP (200 cpm/pmole) + 1 nmole of ^{3}H-dTTP (870 cpm/pmole) or 1.5 nmoles of ^{32}P-ATP + 5 nmoles of UTP or 5 nmoles of dATP + 1 nmole of ^{3}H-dTTP; all other conditions were as described in Table 1 with dAT copolymer as primer. Where indicated, 1.02 units of holoenzyme or 1.5 units of core RNA polymerase were added.

TABLE 5

CHEMICAL CHARACTERIZATION OF ALKALINE HYDROLYZED PRODUCTS

Additions	Products formed with α^{32}P-UTP + dATP	Products formed with α^{32}P-UTP + ATP
	Norite adsorbable ^{32}P (cpm)	
1. Alkaline hydrolyzed product	1040	10,912
2. 1 + BALP (0.03 unit)	1091	167
3. 1 + BALP (0.075 unit)	891	108
4. 1 + spleen phosphodiesterase (0.8 unit)	1074	---
5. 4 + BALP (0.03 unit)	87	---

Reaction mixtures (0.2 ml) were as described in Table 4 except that all additions were doubled and only holoenzyme was employed. After 20 min at 38°, acid-insoluble material was collected by repeated acid-precipitation and neutralization. The acid-insoluble materials were dissolved in 1.0 ml of 0.5 N NaOH, incubated at 38° for 18 hrs and then cautiously neutralized with solid Dowex 50 (H^{+}) filtered and aliquots treated with enzymes as indicated. The abbreviation BALP refers to bacterial alkaline phosphatase.

hydrolysis of the phosphodiester bond. As summarized in Table 5, this is indeed the case. Similar results (not reported) were obtained with products isolated from reaction mixtures containing α^{32}P-ATP + dTTP. Further substantiation of the dinucleotide structure was obtained by electrophoretic separation of the products. Alkaline hydrolyzed products prepared from poly dAU migrated 44 cm from the origin after 2 hrs at 6000 V in 0.03 M sodium citrate buffer, pH 3.5; U^{32}p peaked at 39 cm; the alkaline product formed from polymers synthesized with ATP + α^{32}P-UTP (A^{32}P) peaked at 20 cm. ^{32}P was not detected in other regions of the electropherogram. Further indications of the alternating sequence of ribo and deoxynucleotides in the products was obtained by the demonstration that after treatment with spleen phosphodiesterase the ^{32}P present in dA^{32}pUp was recovered as dA^{32}P.

Susceptibility of the polymeric products to attack by various nucleases was examined. The products used and extent of hydrolysis (in %) were as follows: poly (dTpA), RNase (10 μg) <5; DNase (2.5 μg) 55; DNase (5 μg) 90. In the case of the product poly (dApU) degradation with nucleases was: RNase (10 μg) 93; (2.5 μg) 56; DNase (5 μg) 89. Degradation of the products labeled with ^{32}P-dTTP + ATP by the combined action of pancreatic DNase and venom phosphodiesterase yielded ^{32}P-dTMP which was identified as 5'-dTMP based on its susceptibility to 5'-nucleotidase and its separation from 5'-ribomononucleotides after chromatography in borate-isopropanol-NH_3 (1).

Physical Characterization of the Product. The products formed with α^{32}P-ATP + dTTP and dATP + α^{32}P UTP by RNA polymerase using d(AT) copolymer as template were examined by density gradient centrifugation in Cs_2SO_4 after denaturation in the presence of HCHO (Fig 1). The majority of the product banded at a density higher than dAT copolymer and considerably lower than poly rAU. In HCHO-Cs_2SO_4 gradients dAT copolymer banded at a density of 1.45; the poly (AdT) product formed banded at a density of 1.51 with a marked shoulder at 1.47. The material observed at a density of 1.51 with poly (AdT) is in agreement with the density reported by Chamberlin for poly (AdU) (11). The material banding at a density of 1.47 contains ribonucleotides covalently attached to dAT copolymer primer and is discussed in greater detail in the accompanying manuscript (12).

DISCUSSION

The results presented above indicate that DNA-dependent RNA polymerase of E. coli can incorporate deoxynucleotides and yield mixed polymers containing ribo and deoxynucleotides covalently linked. The reaction is stimulated by Mn^{++} and in this respect is similar to findings with DNA polymerase I (2) and polynucleotide phosphorylase (15). It is evident that the DNA-dependent RNA polymerase of E. coli acts less efficiently with deoxynucleoside triphosphates as substrates. This low order of activity observed with deoxynucleotides is rifampicin and streptolydigin

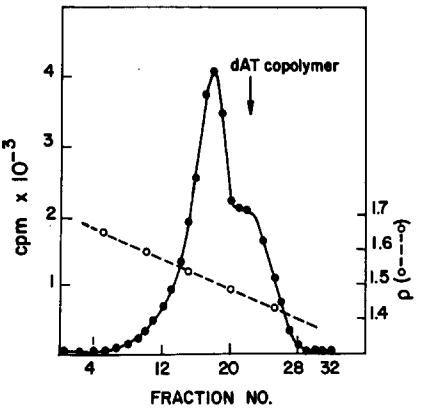

Figure 1. Isopycnic Banding of poly (AdT) Product in Cs_2SO_4. Reaction mixtures were as described in Table 4 but increased in volume to 0.2 ml. After 20 min at 38°, 0.1 ml aliquot received 20 μmoles of EDTA, 0.05 ml of 10 M HCHO, 250 μmoles of sodium phosphate buffer, pH 7.0, 0.02 ml of 10% sarkosyl and was diluted to 2.5 ml with H_2O. The mixture was heated at 75° for 10 min and then cooled. The mixture was treated with solid Cs_2SO_4 to give a density of 1.5. The mixtures were overlayed with mineral oil and centrifuged for 48 hrs in polyallomer tubes at 36,000 rpm at 20°. The material was collected from a hole pierced at the bottom of the tube, refractive indices measured and acid-precipitated. The recovery of radioactivity was 90%. The arrow denotes the position of an internal ^{3}H-dAT copolymer marker which was included in the reaction mixture prior to the HCHO treatment.

sensitive suggesting these results are not due to the presence of DNA polymerases I, II or III.

The utilization of deoxynucleotides by RNA polymerase prompted an examination of its ability to initiate polynucleotide chains with dATP. To date only equivocal results have been obtained utilizing poly dT as template. If chain initiation with dATP occurs, the reaction is far less efficient than initiation of chains with ATP.

Since a number of polymerases can incorporate both ribo and deoxynucleotide we can expect to detect in various polymers the covalent linkage of ribo and deoxynucleotides. The presence of enzymes such as ribonuclease H (16) and ribonuclease III (17) in biological systems indicates that there are mechanisms for removing ribonucleotides in DNA-like polymers. It is tempting to speculate that a similar system for removing deoxynucleotides from RNA may be observed in the future.

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