SPECIFICITY OF RNA CHAIN INITIATION BY BACTERIOPHAGE T7-INDUCED RNA POLYMERASE

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SUMMARY: Analysis of the nucleotide sequence at the 5'-triphosphate termini of RNA chains synthesized by T7 RNA polymerase from T7 DNA template indicates that nearly all RNA chains synthesized in this polymerase reaction contain the sequence, pppGpGp. In addition, studies carried out on T7 DNA-dependent $^{32}PP_i$ exchange into ribonucleoside triphosphates suggest that immediately following the guanine residues at the 5'-end of RNA formed in the T7 RNA polymerase reaction, there is one or more adenine residues. These results indicate a high degree of specificity of initiation of RNA synthesis by T7 RNA polymerase.

INTRODUCTION: Infection of Escherichia coli with bacteriophage T3 or T7 leads to the induction of a new phage specific DNA dependent RNA polymerase (1-5). Both of these phage coded enzymes have been shown to be physically and biochemically distinct from the E. coli RNA polymerase, and are required for the expression of late regions of the respective bacteriophage genome (1-11). One of the striking properties of both T3 and T7 RNA polymerases is their template specificity (1-4); both enzymes copy the homologous phage DNA efficiently, initiate RNA chains only with GTP (4,5) and are inactive with a wide variety of other native DNA templates. In view of the high template DNA specificity of T3 and T7 RNA polymerases, it is possible that there are unique nucleotide sequences or structural alteration(s) (or both) in T3 and T7 DNAs which are recognized by these phage polymerases to initiate RNA chains. If this is true, we would expect the 5'-triphosphate termini of RNA chains to have similar nucleotide sequences.

A previous report from this laboratory (12) has demonstrated that all RNA chains formed in the T3 RNA polymerase reaction directed by native T3 DNA as

template contain the sequence $pppGp(Gp)_nAp....$ at the 5'-terminus, indicating a high degree of specificity of initiation of RNA chains.

The present communication presents evidence which demonstrates a high degree of specificity of initiation of RNA synthesis by T7 RNA polymerase.

MATERIALS AND METHODS: The procedure for isolation of T7 RNA polymerase was similar to that for T3 RNA polymerase as described from this laboratory (9). The final specific activity of the phosphocellulose fraction (9) was 47,500 units per mg protein under standard assay conditions as described under legend to Table I. In this assay, one unit of T7 RNA polymerase promotes the incorporation of 1 nmol of [α-^{32}P] UMP after 15 min at 37°. The final preparation of T7 RNA polymerase was free of detectable exonuclease or endonuclease activities on RNA and DNA. In addition, the enzyme preparation was also free of detectable E. coli RNA polymerase, nucleoside triphosphatase, nucleoside diphosphate kinase and polynucleotide phosphorylase activities. The assays for these enzyme activities were carried out as described previously (9). The sources of all other reagents were as described in previous communications from this laboratory (9-12).

RESULTS

Determination of the Penultimate Nucleotide at the 5'-Triphosphate End of RNA Synthesized in T7 RNA Polymerase Reaction - T7 RNA polymerase, upon copying T7 DNA, initiates RNA chains only with GTP, and the initial guanine nucleotide retains the triphosphate termini (5). In view of this specificity of the initial nucleotide incorporated in RNA chain by T7 RNA polymerase, the question arises whether this specificity extends beyond the first nucleotide. For this purpose we determined the dinucleotide frequency at the 5'-triphosphate end of T 7 RNA polymerase products. These experiments were carried out as follows. Nucleoside triphosphates, labeled with ^{32}P in the α-position were incorporated into RNA products, which were then subjected to alkaline hydrolysis. The ^{32}P-labeled nucleotide adjacent to the triphosphate end was hydrolyzed with alkali and the ^{32}P was transferred to the adjacent guanosine-5'-terminal triphosphate ends, yielding a ^{32}P-labeled guanosine tetraphosphate, $pppGp^*$ as shown below.

$$pppG\overset{*}{p}XpYpZp\text{-----} \xrightarrow{OH^-} pppG\overset{*}{p} + {}^{32}\text{P-nucleoside monophosphates}$$

RNA was synthesized in four separate reaction mixtures, each containing a different α-^{32}P-labeled nucleoside triphosphate. Guanosine tetraphosphate was isolated after alkaline hydrolysis followed by high voltage electrophoretic separation. [γ-^{32}P] GTP was used in a fifth separate reaction mixture in order

to identify and quantitate the amount of pppGp formed. The tetraphosphate region of the electropherograms was analyzed for ^{32}P, and the relative contribution of ^{32}P derived from each of the four nucleoside triphosphates (present originally as X in the reaction shown above) was calculated. The results, summarized in Table I, clearly indicate that among the four polymerase reactions that contained α-^{32}P labeled nucleoside triphosphates, ^{32}P-labeled pppGp was formed only in reactions that contained [α-^{32}P] GTP. These results clearly demonstrate that GMP was the unique nucleotide present adjacent to the 5'-triphosphate-ended GTP in RNA chains formed in the T7 RNA polymerase reaction. The molar amount of ^{32}P found in the pppGp region in the electropherogram derived from reaction mixtures containing [α-^{32}P]GTP was approximately twice that obtained from reactions containing [γ-^{32}P]GTP (Table 1). This is expected, since in the former case the ^{32}P composition of the dinucleotide sequence is $pp\overset{*}{p}G\overset{*}{p}$, while in the later case it is $\overset{*}{p}ppGp$.

Further Studies on the Specificity of RNA Chain Initiation by T7 RNA Polymerase by DNA-dependent $^{32}PP_i$ Exchange Reactions - RNA polymerases catalyze DNA-dependent $^{32}PP_i$ exchange reaction with nucleoside triphosphates even in the absence of RNA chain elongation if only the first phosphodiester bond is allowed to form (12-14). Thus the $^{32}PP_i$ exchange reaction with a single ribonucleoside triphosphate in the presence of catalytic amounts of initiating nucleotide can be used as a tool to study specificity in initiation of transcription by RNA polymerases (12-14).

T7 RNA polymerase catalyzes a T7 DNA-dependent $^{32}PP_i$ exchange into ribonucleoside triphosphates. Incorporation of $^{32}PP_i$ into a Norit-adsorbable form was absolutely dependent on T7 RNA polymerase, Mg^{++} and T7 DNA; omission of DNA or replacement of T7 DNA with T4 DNA or calf thymus DNA led to no detectable $^{32}PP_i$ incorporation (data not shown). Thus the specificity for template DNA for $^{32}PP_i$ exchange reaction is similar to that for the polymerization reaction catalyzed by T7 RNA polymerase (1,5). The ribonucleoside triphosphate requirements for pyrophosphate exchange reaction is shown in Table II. It is evident that GTP is absolutely required for the $^{32}PP_i$ exchange reaction. This result

TABLE I

Analysis of Nucleotide Adjacent to 5'-Guanosine Triphosphate End

^{32}P-labeled nucleotide in polymerase reaction	Possible dinucleotide sequence at 5'-triphosphate end	^{32}P content in guanosine tetraphosphate region
		(pmoles)
[γ-^{32}P] GTP	$\overset{*}{p}$pppGpX	12.6
[α-^{32}P] GTP	pp$\overset{*}{p}$G$\overset{*}{p}$G	26.8
[α-^{32}P] ATP	pppG$\overset{*}{p}$A	0.5
[α-^{32}P] UTP	pppG$\overset{*}{p}$U	< 0.3
[α-^{32}P] CTP	pppG$\overset{*}{p}$C	< 0.3

Table I Each reaction mixture (0.25 ml) contained 50 mM Tris-HCl buffer (pH 7.8), 20 mM $MgCl_2$, 4 mM dithiothreitol, 20 nmoles (as deoxynucleotide residues) of T7 DNA, 80 nmoles each of UTP, CTP, ATP and GTP, and 14 units of T7 RNA polymerase. Five such separate reaction mixtures were prepared; four of these reaction mixtures contained one of each of the four α-^{32}P labeled ribonucleoside triphosphates while the fifth contained [γ-^{32}P] GTP. The specific radioactivities of all ^{32}P-labeled triphosphates were adjusted to 2 x 10^9 cpm per µmole. After incubation at 37° for 15 min, pancreatic DNase (5 µg) was added to each reaction mixture and the mixtures were incubated for an additional 10 min at 37°. Reaction mixtures were then chilled in ice; 200 µg of E. coli tRNA was added as carrier, followed by the addition of 0.2 ml of 7% $HClO_4$. The resulting precipitate was collected by centrifugation and was dissolved in 0.4 ml of ice-cold 0.1 M sodium pyrophosphate solution containing 10 mM EDTA (pH 7.5); this was followed by the addition of 4 ml of 5% CCl_3COOH solution. The resulting acid-insoluble pellet was collected by centrifugation, and the washing procedure was repeated two more times. The final pellet was dissolved in 1 ml of 0.32 N KOH and incubated for 18 hours at 37°. The hydrolyzed mixture was neutralized with solid Dowex 50 (H^+) to pH 7.0, and the supernatant solution was evaporated to dryness. The residue was dissolved in 50 µl of water, and the resulting solution was subjected to paper electrophoresis on a DEAE paper in 0.05 M sodium citrate buffer, (pH 4.0) at 4000 volts for 3 hours as described by Konrad et al. (16). Under the above conditions of electrophoresis, guanosine tetraphosphate, pppGp, moved well behind the (2',3') nucleoside monophosphates with no evidence of cross contamination. The radioactivity corresponding to the tetraphosphate region, was cut into 0.5 cm strips and counted in a liquid scintillation counter.

is in keeping with the observations reported by Chamberlin and Ring (5) that in the T7 RNA polymerase reaction, RNA chains are initiated exclusively with GTP. It should be noted that GTP alone can support the $^{32}PP_i$ exchange reaction. The rate of incorporation of $^{32}PP_i$ into ribonucleoside triphosphates did not sub-

stantially increase if in addition to GTP, any one of the other three ribonucleoside triphosphates either alone or in combination was added to the polymerase reaction.

In order to identify the composition of the 5'-nucleotide sequence of RNA formed in the T7 RNA polymerase reaction, we determined whether a T7 DNA-dependent $^{32}PP_i$ exchange reaction occurred with any one of the other three ribonucleoside triphosphates when the reaction was carried out in the presence of GTP plus a single other ribonucleoside triphosphate (ATP, CTP, or UTP). An exchange reaction with any one of these nucleotides would indicate that this nucleotide is adjacent to the guanine nucleotides at the 5'-end. These experiments were

Influence of Nucleoside Triphosphates on the $^{32}PP_i$ Exchange reaction

Additions	Norit-adsorbable ^{32}P
	(nmoles)
ATP + UTP + CTP + GTP	0.24
ATP + UTP + CTP	< 0.01
ATP + UTP + GTP	0.25
ATP + CTP + GTP	0.23
CTP + UTP + GTP	0.22
(GTP + ATP) or (GTP + CTP) or (GTP + UTP)	0.22-0.23
GTP alone	0.23
(ATP + CTP) or (ATP + UTP) or (CTP + UTP)	< 0.01

TABLE II

Reaction mixtures (0.25 ml) contained 50 mM Tris-HCl buffer (pH 7.8), 20 mM $MgCl_2$, 4 mM dithiothreitol, 20 µg of dialyzed bovine serum albumin, 20 nmoles of T7 DNA, 100 nmoles of $^{32}PP_i$ (4×10^7 cpm/ mole), 40 nmoles each of UTP, CTP, ATP, and GTP as indicated in the table, and 15 units of T7 RNA polymerase. After incubation at 37° for 30 min, the reaction was terminated by adding 0.5 ml of 0.2 M EDTA, 0.2 ml of 0.1 M PP_i (adjusted to pH 6.0 with KH_2PO_4) and 0.2 ml of 10% suspension of Norit. The incorporation of ^{32}P into a Norit-adsorbable form was subsequently measured by the method of Krakow and Fronk (13) as described in a previous communication from this laboratory (9).

carried out as follows. Three separate reaction mixtures, each one containing GTP plus any one of the other three ribonucleoside triphosphates, ATP, UTP, or

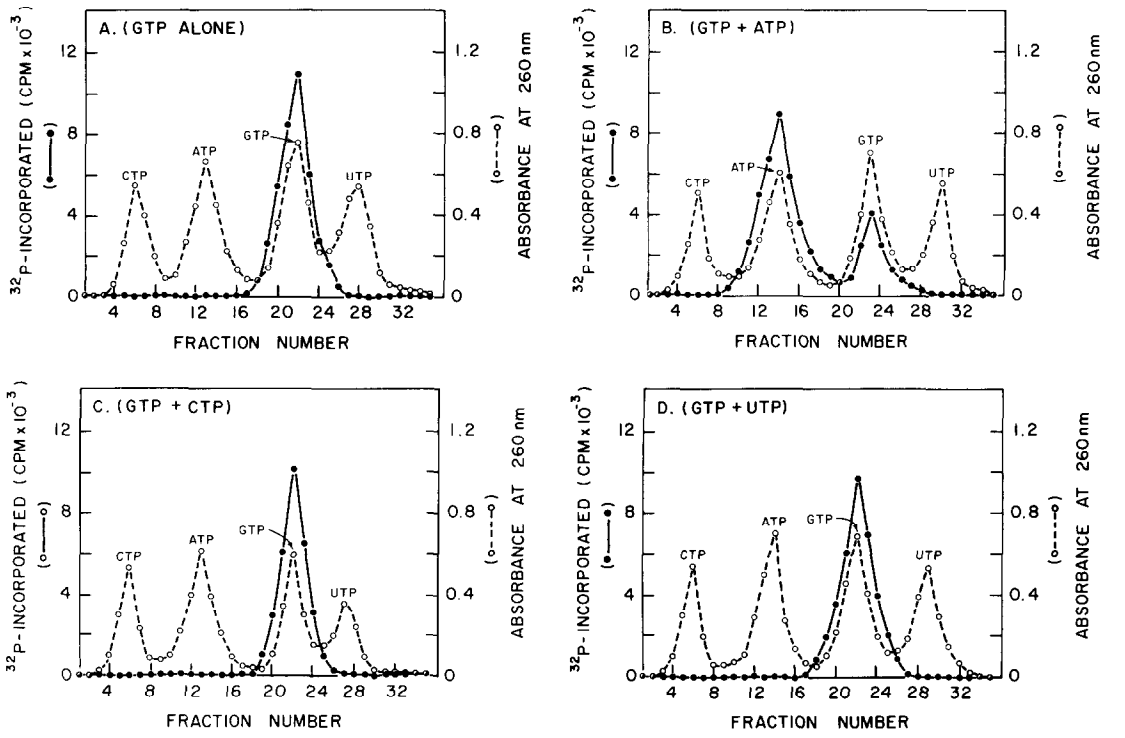

Fig. 1. Identification of products of T7 DNA-dependent $^{32}PP_i$ exchange reaction. Four reaction mixtures (0.2 ml each), each containing different combinations of ribonucleoside triphosphates were prepared as follows: A, GTP alone, B, GTP + ATP; C, GTP + CTP; D, GTP + UTP. The other conditions of the assay were as described under legend to Table II. The specific radioactivity of $^{32}PP_i$ was 2×10^5 cpm per nmole and 15 units of T7 RNA polymerase were added to each reaction mixture. After incubation at 37° for 30 min, reactions were halted and nucleotides were adsorbed to Norit. The Norit was washed with 10 mM PP_i, pH 6.0 and subsequently twice with water. The radioactive nucleotides were then eluted from Norit with 1 N NH_3 in 50% ethanol solution. The eluate was evaporated to dryness and taken up in a small volume of water. After adding GTP, ATP, CTP, and UTP (2 μmoles of each) to each reaction mixture as carrier, each solution was applied to a column of Dowex 1 (Cl) (1 cm^2 x 8 cm); 100-200 mesh; 2% cross-linked) which had previously been washed extensively with water. Each column was washed successively with 150-ml volumes of 10 mM HCl, containing increasing concentrations of LiCl as follows: (a) none, (b) 40 mM LiCl; (c) 80 mM LiCl; (d) 0.12 M LiCl; (e) 0.15 M LiCl. Fractions of 10 ml were collected. The elution profiles of each ribonucleoside triphosphate were determined by measuring absorbance of effluents at 260 nm and at 280 nm. The above separation procedure completely resolved CTP, ATP, GTP, and UTP from one another and were eluted in this order from the column. Aliquots of 1 ml were counted for ^{32}P in Bray's solution in a liquid scintillation counter. The results are expressed in the above figure as the total ^{32}P radioactivity present per ml of each fraction. The recovery of total radioactivity from each chromatogram was better than 90%.

CTP were prepared. In a fourth reaction mixture, the exchange reaction was carried out with GTP alone. The product of the exchange reaction was identified in each case by Dowex 1 (Cl) chromatography (Fig. 1). With GTP as the sole nucleoside triphosphate present, all incorporated ^{32}P cochromatographed with GTP (Fig. 1A). When the exchange reaction was carried out with (GTP + CTP) or (GTP + UTP), the incorporated ^{32}P again cochromatographed with GTP, no ^{32}P-labeled CTP or UTP was detected (Fig. 1, C&D). In contrast, when the exchange reaction was carried out in the presence of GTP + ATP, a substantial amount of incorporated ^{32}P cochromatographed with ATP, while some ^{32}P appeared also in the GTP region (Fig. 1B). On a quantitative basis, nearly 75% of the incorporated ^{32}P cochromatographed with ATP, the remaining 25% with GTP. These results are consistent with the conclusion that immediately following the guanine residues at the 5'-end of the RNA chains formed in the T7 RNA polymerase reaction, there is one or more adenine residues.

DISCUSSION: Results presented in this communication clearly demonstrate a high degree of specificity of RNA chain initiation by T7 RNA polymerase. Nearly all RNA chains synthesized in this polymerase system are initiated with the sequence, pppGpGp. Furthermore the data on the $^{32}PP_i$ exchange studies presented above suggest that one or more adenine residues are present immediately following the guanine residues at the 5'-end.

Recent reports by Golomb and Chamberlin (15) and by Niles et al. (7) have clearly demonstrated that six discrete size classes of RNAs comprising seven major RNA species are synthesized by T7 RNA polymerase from a T7 DNA template. It is not yet known whether each RNA species contains an identical initiation sequence at the 5'-end. Isolation of each of these RNA species, followed by direct nucleotide sequence analysis at the 5'-end will be necessary to answer this question.

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