NUCLEOTIDE SEQUENCES AT THE 5'-TERMINI OF IN VITRO SYNTHESIZED RNA

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The nucleotide sequences at the 5'-termini of RNA formed in the DNA-dependent RNA polymerase reaction using a calf thymus DNA template were studied. Pancreatic or T_1 ribonuclease digestion of RNA bearing a ^{32}P-label at the 5'-termini, followed by fractionation of radioactive terminal fragments on DEAE-cellulose in 7 M urea did not show the presence of unique sequences in the nucleotide chain following the first purine nucleotide at the 5'-terminus. The distribution of sequences was independent of the secondary structure of the template DNA.

It is widely believed that DNA-dependent RNA polymerase binds to DNA at specific sites prior to the initiation of transcription (1-6). If specific sites, unique nucleotide sequences, for binding of the enzyme exist, then they may be reflected in the sequences near the initiation points (5'-termini) of synthesized RNA chains. An additional reason for studying the 5'-terminal sequences of RNA arises from their possible role in translation.

The RNA chains formed by RNA polymerase contain ribonucleoside triphosphates at their initiation points (7,8), thus label from γ-^{32}P-nucleoside triphosphates is incorporated only at the 5'-terminus. It has already been shown that RNA chains are initiated almost exclusively by purine nucleoside triphosphates (7-11).

In this communication we report the 5'-terminal nucleotide sequences of RNA formed in the RNA polymerase reaction with native

or denatured calf thymus DNA as template.

MATERIALS

DNA-dependent RNA polymerase (EC 2.7.7.6) was purified from Escherichia coli strain B (H) using DEAE-cellulose chromatography, ammonium sulfate fractionation, hydroxylapatite chromatography and DEAE-Sephadex chromatography, successively (12). Nucleic acids, RNase, DNase and polynucleotide phosphorylase were either absent or present in negligible amounts and the specific activity of the final preparation was approximately 4000 units/mg protein (according to the method of Chamberlin and Berg (13)).

ATP and GTP labeled with ^{32}P in the γ-phosphorus (specific activities, 1 to 2 $\times$ 10^9 cpm/μmole) were prepared by the procedure of Glynn and Chappell (14) and further purified by chromatography on Dowex 1. Calf thymus DNA and pancreatic ribonuclease (RNase) were obtained from Worthington Biochemicals Corp. RNase T_1 was purchased from Sankyo Co. Ltd.

RESULTS AND DISCUSSION

The effect of the secondary structure of a DNA template on chain initiation has been reported by several groups (8-11). Fig. 1 shows the results of a similar experiment with our RNA polymerase system. When native DNA is used as template the rate of incorporation of γ-^{32}P-ATP and GTP is initially rapid and then reaches a plateau after about 10 min of incubation, while with heat-denatured DNA as template chain initiation continues for more than 30 min and is several times greater than that with native DNA. It is therefore expected that initiation on the denatured DNA template is somewhat different from that on the native one. Accordingly we analyzed RNAs formed on both native and denatured DNA.

Fig. 2 (A) shows an elution profile of an RNase T_1 digest of

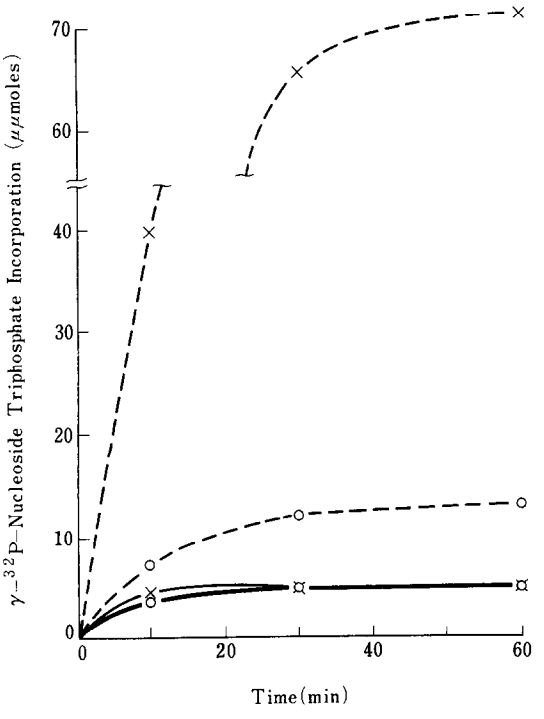

Fig. 1. Influence of the secondary structure of the DNA template on the initiation of RNA synthesis.
Each reaction mixture (0.2 ml) contained Tris-HCl buffer, pH 7.6, 24 µmoles; $Mg(Ac)_2$, 1.2 µmoles; $MnCl_2$, 0.4 µmole; dithiothreitol, 0.8 µmole; ATP, GTP, UTP and CTP 0.04 µmole each with either the ATP or the GTP labeled with ^{32}P in the γ-phosphorus for measurement of ATP (O) and GTP (×) initiation respectively; native (——) or heat-denatured (----) calf thymus DNA, 16 µg; and 16 units of RNA polymerase. The reactions were incubated at 37°C, and 1 mg of yeast RNA was added, followed by 0.2 ml of 7 % $HClO_4$ at the indicated times. The acid-insoluble pellet was washed essentially according to the procedure of Maitra et al. (11) and the radioactivity was counted in toluene-POPOP-PPO scintillation fluid in a Beckmann LS 200B instrument.

γ-^{32}P-ATP-labeled RNA from a DEAE-cellulose column (18). Five major radioactive peaks were observed. The first radioactive peak appeared between the fifth and the sixth ultraviolet-absorbing peaks, consisting of penta- and hexanucleotides respectively. A dinucleoside pentaphosphate of the form pppApGp is most consistent with the chromatographic behavior (15-17) and enzymatic formation of the labeled fragment. Similarly, we assign the second peak to pppApXpGp*, the third to $pppAp(Xp)_2Gp$ and the fourth to $pppAp(Xp)_3Gp$. Finally, by elevating the NaCl concentration to 1 M, fragments larger than a hexanucleotide ($pppAp(Xp)_{\geq 4}Gp$) were eluted from the column. The pppApXpGp fraction was desalted by adsorption on a small DEAE-cellulose column, elution with triethyl-

*X = A or Py

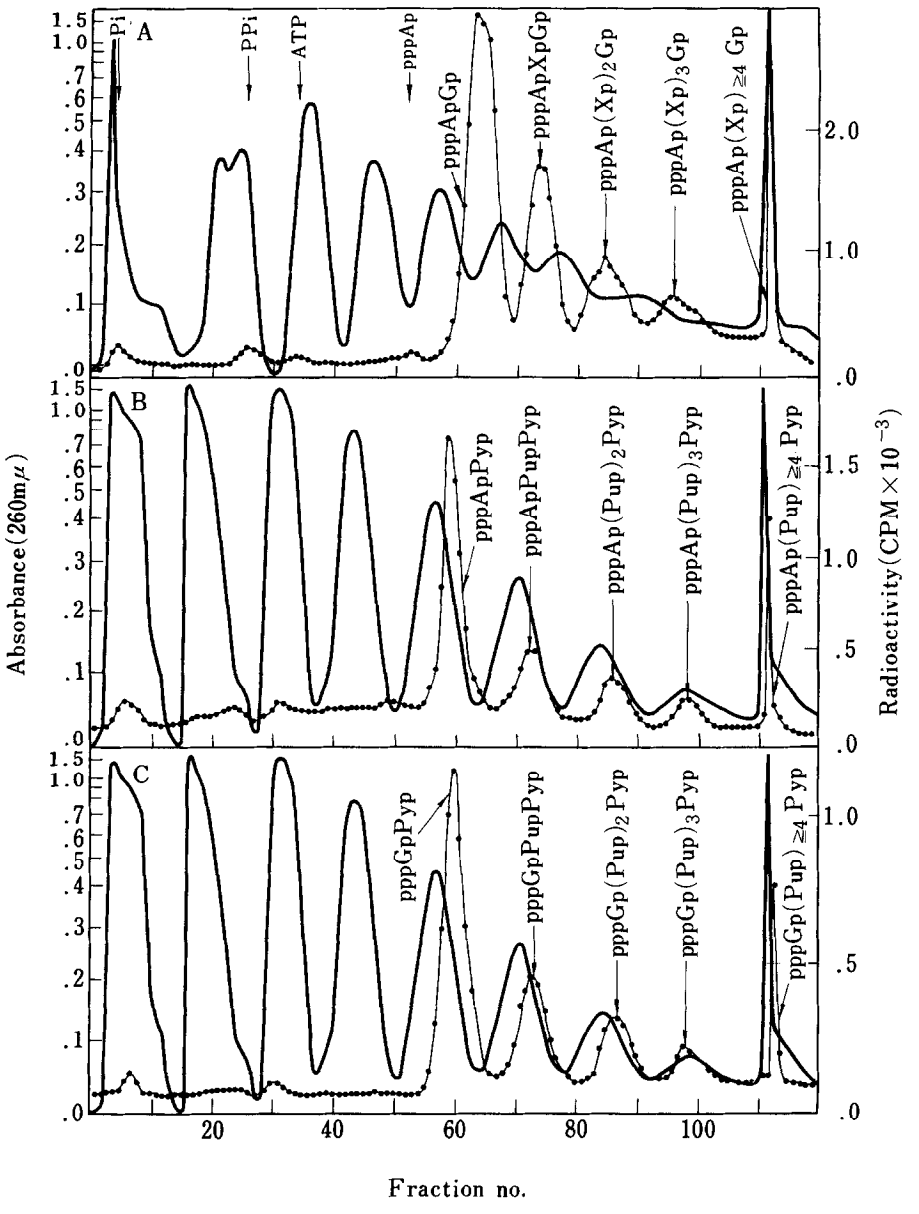

Fig. 2. Chromatography of T_1 and pancreatic RNase digests of RNA labeled with ^{32}P at the 5'-termini.
RNA was synthesized in a 4 ml reaction mixture with native calf thymus DNA as template. After a 10 min incubation at 37°C, 0.5 mg of yeast RNA was added and the mixture extracted with SDS-phenol. RNA was precipitated from the upper layer by adding ethanol and washed as described in the legend to Fig. 1. The RNase treatment was carried out with 2.5 μg of enzyme in 0.2 ml of 0.05 M Tris-HCl, pH 7.6, containing 0.001 M EDTA. After incubation at 37°C for 16 hr, the RNase T_1 digest was treated with 20 μl of 1 N HCl for 6 hr at 0°C to cleave cyclic phosphates. In the case of the

pancreatic RNase digestion, the acid hydrolysis was omitted. Chromatography on DEAE-cellulose (TLC, Serva) essentially followed the method described by Tener (18). The hydrolysate was charged on a column (0.06 cm^2 × 17 cm) and eluted with a gradient of NaCl concentration formed from 20 ml of 7 M urea containing 0.02 M Tris-HCl, pH 7.8 and 40 ml of 7 M urea containing 0.15 M NaCl and 0.02 M Tris-HCl, pH 7.8. The column was connected to a recording spectrophotometer to monitor absorbance at 260 mμ (——). The radioactivity of each fraction (0.5 ml) was measured in toluene-POPOP-PPO scintillation fluid (—●—). The elution position of orthophosphate, pyrophosphate, ATP, and adenosine tetraphosphate was determined by adding the respective ^{32}P-labeled compounds to the optical density marker.
(A): T_1 RNase digest of RNA containing γ-^{32}P-ATP at 5'-termini.
(B): Pancreatic RNase digest of RNA containing γ-^{32}P-ATP at 5'-termini.
(C): Pancreatic RNase digest of RNA containing γ-^{32}P-GTP at 5'-termini.

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ammonium bicarbonate and evaporation (18). Treatment of this fraction with pancreatic RNase followed by separation on DEAE-cellulose with a linear gradient produced from 0.035 M NaCl in 7 M urea, pH 3.8 (adjusted with formic acid) and 0.12 M NaCl in 7 M urea, pH 3.8 (19) yielded three radioactive peaks, which are expected to be pppApCp, pppApUp and pppApApGp. The former two should be derived from pppApCpGp and pppApUpGp respectively and represent the amount of these fragments.

The chromatographic pattern of a pancreatic RNase digest of RNA containing γ-^{32}P-ATP at the 5'-termini is presented in Fig. 2 (B). Five major radioactive peaks appeared and are expected to be pppApPyp, pppApPupPyp, pppAp$(Pup)_2$Pyp, pppAp$(Pup)_3$Pyp and pppAp$(Pup)_{\geq 4}$Pyp as shown in the figure. The pppApPyp fraction was analyzed into pppApCp and pppApUp portions by DEAE-cellulose chromatography at pH 3.8 as described above. The fraction corresponding to the pppApPupPyp-region was desalted, treated with RNase T_1 and the amount of pppApGpPyp and pppApApPyp were determined by means of DEAE-cellulose chromatography at pH 7.8. A pancreatic RNase digest of RNA containing ^{32}P-GTP termini was analyzed in the same manner. The resulting pattern of optical

density and ^{32}P distribution is presented in Fig. 2 (C). The 5'-terminal fragments from RNA synthesized on heat-denatured calf thymus DNA was also analyzed by the same chromatographic techniques.

Experiments were repeated using different polymerase preparations and very similar results were obtained consistently. The percentage contribution of each peak to the total radioactivity

Table I The 5'-terminal fragments from *in vitro* synthesized RNA by T_1 or pancreatic RNase treatment

	Calf thymus DNA	Native	Denatured*	Denatured*
	Reaction time (min)	10	10	60
	Fragment**	Distribution (%)		
T_1 RNase	pppApGp	28.4	28.8	33.6
	pppApCpGp	4.7	2.8	15.9
	pppApUpGp	6.6	6.4	
	pppApApGp	4.7	5.0	
	pppAp(Xp)$_2$Gp	11.4	10.1	11.4
	pppAp(Xp)$_3$Gp	10.0	7.7	7.8
	pppAp(Xp)$_{\geq 4}$Gp	34.3	39.0	31.4
Pancreatic RNase	pppApCp	20.7	14.0	43.5
	pppApUp	29.1	31.8	
	pppApGpPyp	10.1	10.0	22.0
	pppApApPyp	5.4	7.7	
	pppAp(Pup)$_2$Pyp	9.9	13.0	13.6
	pppAp(Pup)$_3$Pyp	6.8	8.8	8.2
	pppAp(Pup)$_{\geq 4}$Pyp	17.8	14.7	12.7
	pppGpCp	18.9	19.9	—
	pppGpUp	23.4	24.1	—
	pppGpPupPyp	19.5	20.9	—
	pppGp(Pup)$_2$Pyp	15.3	14.7	—
	pppGp(Pup)$_3$Pyp	9.3	9.3	—
	pppGp(Pup)$_{\geq 4}$Pyp	13.6	11.0	—

*RNA synthesized on denatured DNA was recovered from 1.0 ml of the reaction mixture after 10 and 60 min incubation.
**X = A or Py.

is listed in Table I. It is evident that the distributions of fragments from RNAs which have been synthesized on native and denatured DNA are essentially the same, even after a prolonged incubation (60 min) with denatured DNA. As shown in Fig. 2 and Table I, pancreatic RNase digestion of RNA terminally labeled with either ATP or GTP yields essentially the same pattern. This suggests that when RNA chains are initiated by either ATP or GTP, the following oligonucleotide sequences are not specified. From the results shown in Table I, the distribution of the initial trinucleotide sequences was calculated and is presented in Table II. As seen in the table, there is no predominant unique sequence, however, a slight preference for initiation by purine-

Table II The initial trinucleotide sequences at 5'-termini of RNA formed in the RNA polymerase reaction

	Native DNA	Denatured DNA*		Native DNA	Denatured DNA*
A U U, A U C, A U A	22.6	25.4	G U Py, G U Pu	23.4	24.1
A U G	6.6	6.4			
A C U, A C C, A C A	16.0	11.2	G C Py, G C Pu	18.9	19.9
A C G	4.7	2.8			
A A U, A A C	5.4	7.7	G Pu Py	19.5	20.9
A G U, A G C	10.1	10.0			
A A A	11.7	12.6			
A A G	4.7	5.0	G Pu Pu	38.2	35.0
A G A, A G G	18.3	18.9			

*Calculated from the 10 min reaction data in Table I.

rich sequences, such as pppApGpPup, pppApApAp and pppGpPupPup, was observed. Although DNA-dependent RNA polymerase initiates its reaction exclusively by purine nucleoside triphosphates, the following nucleotide sequences are rather random.

It is not clear whether the initiation by random sequences observed here is a general feature or is due to the origin of the template DNA in a species other than E. coli, the enzyme source. The same analysis of RNA synthesized in the presence of DNA from other sources is in progress.

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