Short RNA chains synthesized at low pH are initiated at promoter sites

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ABSTRACT

Under non optimal conditions- either with limiting substrate concentrations (1) or at low pH (2)- RNA polymerase of Escherichia coli synthesizes very short RNA chains. By sequencing one RNA species synthesized at pH 5.8 upon T7 DNA we were able to demonstrate that under these conditions transcription is initiated at a normal promoter site (here A1) but however is terminated soon afterwards at specific artificial sites not used in vivo.

INTRODUCTION

Under optimal in vitro conditions the RNA synthesized by the DNA dependent RNA polymerase (E.C.2.7.7.6.) has a chain length of several thousand nucleotides (3,4). Under conditions of low ionic strength this chain length is not substantially reduced (5); however, the polymerase is not able to reinitiate transcription after termination (6).

For sequence analysis it is often desirable to obtain distinct short RNA chains in high yield, i.e. the polymerase should be able to restart synthesis after terminating the RNA chains at discrete sites close to the initiation site. Indeed, such conditions can be adjusted: at a low pH of 5.6-6.0 and at low ionic strength the chain length is reduced to about 20-200 nucleotides (2). These short RNA pieces are each of homogenous size and sequence. On different templates the pattern of RNA's synthesized is different; this is true even for the DNA's of the related phages T3 and T7.

The question arises whether this RNA is initiated at promoter sites and terminated at some artificial points on the

genome, or whether both the start and termination sites or only the start sites are artificially created.

Here we give evidence by sequencing of one RNA species synthesized from T7 DNA that the RNA is initiated at a promoter site - in this case T7-A1 - and terminated at some artificial site.

MATERIALS AND METHODS

RADIOCHEMICALS: ($\alpha^{32}\text{P}$) and ($\gamma^{32}\text{P}$) ribonucleotides were prepared according to Symons (7,8) and Glynn and Chappel (9) respectively.

ENZYMES: DNA dependent RNA polymerase was purified from *Escherichia coli* MRE 600 by standard methods (2). The enzyme was homogenous, free of nuclease activity and saturated with sigma subunit. RNase A, snake venom phosphodiesterase, alkaline phosphatase (Boehringer) and T1 RNase (Calbiochem) were commercial enzymes. Bacteriophage T4 polynucleotide kinase was generously provided by Dr.H.Schaller.

BACTERIOPHAGE AND DNA: Bacteriophage T7 wt was obtained from Dr.F.W.Studier. Phages were grown and purified as described previously (10). DNA was isolated by phenol extraction of CsCl purified bacteriophage T7 and dialysed against 0.01 M Tris/acetate pH 7.5. The DNA had no single stranded breaks as determined by alkaline agarose gel electrophoresis (11).

RNA SYNTHESIS was usually carried out in a volume of 50 μl . The reaction conditions were : 4 mM Mg^{2+} , 12 mM KCl, 0.1 mM of each CTP, UTP and ATP and 20 μM ($\alpha^{32}\text{P}$) GTP (20 Ci/mmol), 30 mM MES (12) buffer (pH 5.85), 150 $\mu\text{g/ml}$ T7 DNA, 50 $\mu\text{g/ml}$ *E. coli* RNA polymerase, 0.8 mM K_2HPO_4 and 0.1 mM ADP. Incubation was at 37°C for 2 hrs. After addition of 10 μg carrier tRNA, the RNA was precipitated with 67% ethanol and washed twice.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA was performed according to Peacock and Dingman (13) in 12.5% polyacrylamide slab gels (14).

ISOLATION OF RNA: After gel electrophoresis the ($\alpha^{32}\text{P}$)labeled RNA was visualized by autoradiography on Kodak RP/54 X-Omat films and eluted by soaking gel pieces in 1% phenol for 5-8 hrs at 22°C. Samples were then desalted through Sephadex G 50 and lyophilized.

DIGESTION OF RNA WITH RNASES A OR T1 was carried out in the presence of 10 μ g carrier tRNA for 10 min (RNase A) or 20 min (RNase T1) in 0.01 M Tris/acetate pH 7.5 with RNA/enzyme ratio of 10:1 (w/w). For partial digestion with snake venom phosphodiesterase oligonucleotides were dephosphorylated using alkaline phosphatase (RNA/enzyme=1000:1) for 15 min at 37°C and protein was removed by extracting once with phenol.

PHOSPHORYLATION OF RNA with T4 polynucleotide kinase was performed according to Pribnow (15) in the presence of (γ -³²P) ATP (250 Ci/m mole) for 10 min at 37°C. After phenol extraction and subsequent dialysis, 10 μ g of carrier tRNA were added and the RNA digested by snake venom phosphodiesterase for 10 min at 22°C using RNA/enzyme ratios of either 10:1 or 20:1 (w/w).

THIN LAYER CHROMATOGRAPHY ON PEI-CELLULOSE: RNase-digested RNA was chromatographed according to Mirzabekov and Griffin (16). The first dimension (bottom to top): 7M urea and 1.5 M Li-formate pH 3.4; the second dimension (left to the right): 7 M urea, 0.7 M LiCl and 10 mM Tris/HCl pH 8.

RESULTS AND DISCUSSION

At pH 5.7 and the ionic strength of 4 mM Mg²⁺ and 12 mM KCl, four main and several minor RNA species are synthesized by E. coli RNA polymerase using T7 DNA as a template. The chain length of the major species are about 25, 70, 80 and 150 nucleotides (2). Reproducible synthesis of these RNA's in adequate yield is obtained only by using intact double stranded DNA without nicks and highly purified RNA polymerase saturated with the sigma subunit and free of nucleases. At a very carefully controlled pH of 5.85 the smallest RNA species becomes the main product (Fig.1). This RNA was separated from the others by polyacrylamide gel electrophoresis (2) and its chain length was estimated (by comparison with tRNA and with oligonucleotides containing 2, 4 and 20 bases (17)) to be between 24-26 nucleotides.

In order to show that this RNA species was initiated at one of the normal T7 promoter sites, its sequence was compared with the 5' ends of the early T7 mRNA's.

Figure 1. Autoradiogram of RNA synthesized in vitro at pH 5.85 with T7 DNA as template. Electrophoresis was carried out in 12.5% polyacrylamide slab gels. Band I was used for sequence analysis. Two identical experiments are shown.

For this the RNA was separately labeled with each of the 4 (α - ^{32}P) ribonucleoside triphosphates and subsequently digested by pancreatic or T1 RNase. The cleavage products were separated by thin layer chromatography on PEI cellulose in two dimensions and the oligonucleotides were sequenced by nearest-neighbour analysis (16).

Typical fingerprints are shown in Figure 2. The oligonucleotide of the 5' end of the RNA did not migrate from the origin of the thin layer chromatography because it contained the 5' triphosphate. It therefore was eluted from the origin of a T1 RNase fingerprint, labeled with bacteriophage T4 polynucleotide kinase in the presence of (γ - ^{32}P)ATP and partially digested with snake venom phosphodiesterase (Fig.3). Its sequence was determined to be pppAUCG and a corresponding pppAU was found after RNase A digestion. All oligonucleotides sequenced so far are listed in table 1. By inspecting the overlapping sequences the following partial sequence can be established:

From the origin of the pancreatic RNase digest chromatography on PEI cellulose two oligonucleotides were eluted: one was the terminal 5'pppAU and the other was a purineoligonucleotide the chain length of which was estimated to be 8-10 nucleotides. They were separated from each other by homochromatography according to Jay et al (18). Before the determination of the whole sequence was completed we learned from Dr.U.Siebenlist (19) that the sequence of the T7 RNA initiated at the A1 promoter is the following:

5'A U C G A G A G G G A C A C G G C G A A U A G C C A U 3'

By comparison of our data with this sequence it is evident that both RNA's are identical, i.e. the small RNA synthesized at low pH on T7 DNA is initiated at the A1 promoter. It does not show sequence similarities to the 5' end of the RNA's initiated at either the T7-A2 or T7-A3 promoters (15).

Furthermore the high requirements for the synthesis of the small RNA's at low pH - as described above - may support our hypothesis that under these conditions the polymerase initiates transcription in all cases effectively only at the normal promoter sites. Since the RNA species formed are much shorter than those found in vivo (more than 1000 nucleotides long (3,4)) synthesis must terminate at artificial sites. From present knowledge several factors may cause the termination of transcription: a) poly A stretches close to the terminating site on the strand of the DNA complementary to the mRNA (20); b) GC rich regions according to the "pause hypothesis" of Dr.W.Gilbert (21); c) certain palindromic structures on the DNA (22); and

Figure 2. Two-dimensional thin layer chromatography of RNase-digested T7 RNA (band I of Fig.1) on PEI cellulose. a) and b) pancreatic RNase-digest labeled a) with (α^{32} P)ATP and b) with (α^{32} P)CTP. c) T1 RNase-digest labeled with (α^{32} P)GTP.

d) sequences similar to the binding site of a promoter, the so-called "Pribnow box" (Dr.H.Schaller,pers.communication). Two of these effects could be active in this - the low pH - case: The stop is preceded by two clusters of GC base pairs within the last 13 nucleotides of the 3' end; the sequence G C G A A U A shows similarities to a promoter binding site

Figure 3. Two-dimensional chromatography on PEI cellulose of an oligonucleotide partially digested with snake venom phosphodiesterase. The oligonucleotide was eluted from the origin of an RNase T1 fingerprint (Fig. 2c), dephosphorylated and labeled at its 5' end by bacteriophage T4 polynucleotide kinase (see methods).

(23). Perhaps both factors or even some unknown others are working together. One has nevertheless to assume that the terminating character of this region is more effective under the low pH conditions.

Further experiments have shown that the oligonucleotide pattern of the RNA with about 80 nucleotides chain length

T1 RNase products Sequence ⁺	Pancreatic RNase products Sequence
1 G(G)	10 C(G)
2 G(A)	11 C(A)
3 G(C)	12 AC(G)
4 AG(A)	13 AGC(G)
5 AG(G)	14 GGC(G)
6 CG(A)	15 GAAU(A)
7 ACACG(G)	16 pppAU
8 AAUAG(C)	17 purine oligonucleotide
9 pppAUCG	

Table 1. Analysis of nucleotides in pancreatic and T1 RNase digest of T7 RNA.
+ The 3' neighbouring nucleotides are shown in parenthesis.
Evaluation of oligonucleotides 1,4-8 and 11-15 is shown in Fig. 2.

contained the pattern of the above 26 nucleotide RNA (data not shown here). This result suggests that RNA synthesis starting from one promoter can halt at several different termination sites under conditions of low pH. On the other hand some RNA's were initiated at different promoter sites: By the use of either (γ - ^{32}P)ATP or (γ - ^{32}P)GTP as substrate different RNA bands were labeled.

The method described above will be useful for the isolation of small RNA's in high yield, for characterisation of promoter sites, or for the evaluation of the mechanism of transcription termination by RNA polymerase.

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