NET SYNTHESIS OF SHORT RNA CHAINS BY *E. COLI* RNA POLYMERASE AT LOW pH

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1. Introduction

Commonly for sequence analysis of DNA either homogeneous pieces of relatively short chain length are isolated or relatively short parts of a genome are transcribed or replicated by suitable enzymes and the products of these reactions are analysed [1,2]. In the latter methods the polymerases are stopped shortly after initiation by supplying only three of the four nucleoside triphosphates as substrates or by reducing the substrate concentration. The transcription of short DNA sequences by DNA-dependent RNA-polymerase without these substrate-dependent stops would be of great help for sequence work since it would allow the enzyme to restart, i.e. there would be net synthesis of RNA.

Here we show that under some non-optimal conditions *E. coli* RNA polymerase synthesizes only short RNA which consists of many copies of distinct RNA chains about 20–200 nucleotides long. Hence with the help of this technique it should be possible to obtain short DNA transcripts of large genomes in high yield for sequence analysis.

2. Materials and methods

E. coli RNA polymerase was purified to homogeneity by standard methods [3,4] and was free of nuclease activity. The assay conditions are given in the legends of the figures.

Polyacrylamide gel electrophoresis was accomplished according to the method of Peacock and Dingman [5], and thin layer chromatography on PEI-cellulose according to Mirzabekov and Griffin [6]. For further details see legends.

3. Results and discussion

The optimal pH for the RNA polymerase is between 7.9 and 8.1 [7]. The size of RNA synthesized in vitro under these conditions with T3, T7 or T4 DNA as template is of 3000 to 7000 nucleotides length [8,9]. Only at relatively high ionic strength under these pH conditions is the enzyme able to reinitiate [10]. However at low pH (5.7–6.0) and low ionic strength (3–12 mM Mg^{2+} and 10–40 mM KCl), the rate of RNA synthesis was much lower, but did not reach a plateau and continued for several hours with a rate of synthesis much lower than at optimal conditions (fig.1a). The chain length of the RNA synthesized under these conditions was below 300 nucleotides as could be shown by double labeling experiments with [γ-^{32}P] ATP and/or [γ-^{32}P] GTP and [^{3}H] UTP. The incorporation of the γ^{32}P-labeled ATP and GTP into RNA did not reach a plateau at low pH in contrast with RNA synthesized at high pH (fig.1b). T3 and T7 DNA as template gave identical results. The analysis of the RNA products on polyacrylamide electrophoresis in slab gels gave several defined bands, the pattern of which differed from template to template (fig.2): the pattern with T3 RNA showed 6–7 main bands with chain lengths between 40–200 nucleotides. Though T3 and T7 are very similar phages, the T7 pattern was quite different from T3: only 4–5 main bands with about 20–200 nucleotides chain lengths were obtained. The 9–10 bands of the T5 phage RNA pattern were much weaker than those of T3 and T7. Their chain lengths were in the range of the T3 RNA. With T4 DNA as template only very little RNA could be obtained at pH 5.7. However at pH 6.0 4–5 small RNAs with chain lengths of about 50–150 nucleotides were synthesized and could be separated on the gels. The

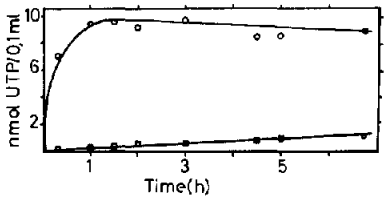

Fig.1a.

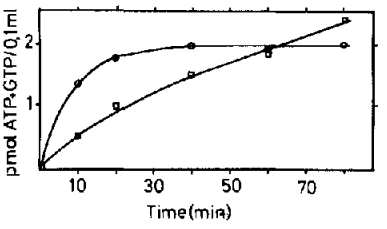

Fig.1b.

Fig.1(a). In vitro RNA synthesis at pH 8 (○) and at pH 6 (□) by *E. coli* RNA polymerase. Assay conditions: 6 mM Mg^{2+}, 24 mM KCl, 1 mM ATP, CTP, GTP and [^{3}H]UTP (5 Ci/mol) each, 100 μg/ml T3 DNA, 60 μg/ml RNA polymerase, 1 mg/ml BSA, 30 mM Tris buffer pH 8 or 30 mM MES buffer [12] pH 6, incubation at 37°C. Aliquots of 0.1 ml were precipitated with 5% TCA and washed through millipore filters. (b) Incorporation of [γ-^{32}P]ATP and [γ-^{32}P]GTP by *E. coli* RNA polymerase at pH 8 (○) and at pH 6 (□). Assay conditions as above except: 50 mM KCl, 0.5 mM CTP, UTP, [γ-^{32}P]ATP, [γ-^{32}P]-GTP (both 37 Ci/mol) each, 35 μg/ml T4 DNA, 15 μg/ml RNA polymerase, 0.1 mM ADP and 0.8 mM K_2HPO_4. Aliquots of 0.1 ml were brought to 0.5% SDS and incubated for 15 min at 50°C. Then the RNA was TCA precipitated and washed as described by Roberts [13].

background and the amount of large size RNA were much higher than with DNAs of the other phages. The chain lengths of the RNAs were estimated using 5s RNA, 4s RNA and an oligoribonucleotide of 8 nucleotides chain length as marker under both denaturing [11] and non denaturing [5] electrophorectic conditions; both methods gave identical patterns. The same bands were obtained by use of both γ^{32}P-labeled ATP and GTP as compared to the incorporation of the α^{32}P-labeled triphosphates. Hence the RNA chains were started with a triphosphate end. Since the labeled bands were obtained when the label was added after some time of preincubation with unlabeled substrates, the polymerase must reinitiate RNA synthesis under these conditions. Therefore it was possible to isolate more than one copy of the different RNA chains per DNA molecule. In the absence of DNA or of Mg^{2+} no RNA synthesis could be observed. The qualitative composition of the band pattern was not influenced by variation of substrate concentration or pH up to 6.0 or of the Mg^{2+} concentration up to 12 mM. By decreasing the pH from 6.0 to 5.7 the yield of total RNA decreased by a factor of about 3. However by increasing the pH above 6.0 and/or the Mg^{2+} concentration above 15 mM and/or the ionic strength above 0.1 M, the amount of large size RNA and the background in the gels increased.

Evidence for the homogeneity of the RNA bands was obtained by fingerprint techniques: the RNA was

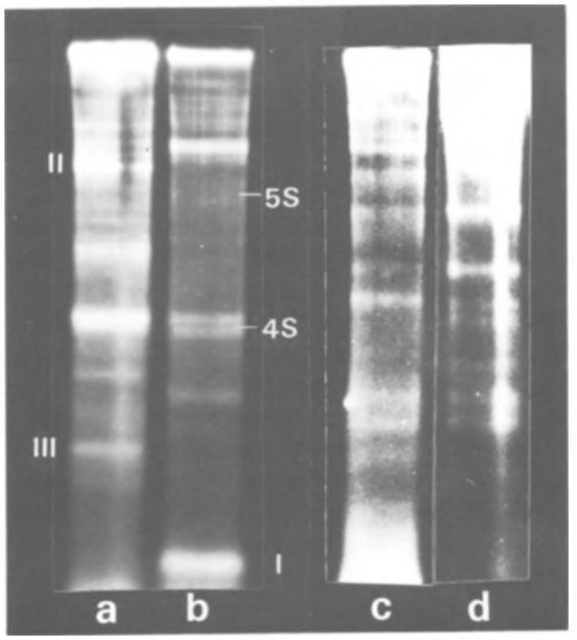

Fig.2. Autoradiogram of in vitro RNA synthesized at low pH with (a) T3 DNA, (b) T7 DNA, (c) T5 DNA and (d) T4 DNA as template. The pH in the case of (a), (b) and (c) was 5.7, in the case of (d) 6.0. Assay conditions: 3 mM Mg^{2+}, 12 mM KCl, 50 μM CTP, GTP, UTP and [α-^{32}P]ATP (20 Ci/mmol) each, 30 mM MES buffer [12] pH 5.7 or pH 6.0, 200 μg/ml DNA, 40 μg/ml *E. coli* RNA polymerase, 0.8 mM K_2HPO_4, 0.1 mM ADP. Incubation at 37°C for 1.5 h. After addition of 40 μg tRNA, the assay was brought to 0.75 M KCl and 0.75% phenol and heated for 2 min to 100°C. RNA was ethanol precipitated and washed twice. Electrophoresis was accomplished in 12.5% polyacrylamide slab gels. Under these conditions 23s and 16s rRNA did not enter the gel. Band I of T7 RNA and bands II and III of T3 RNA were analyzed by fingerprints; see fig.3.

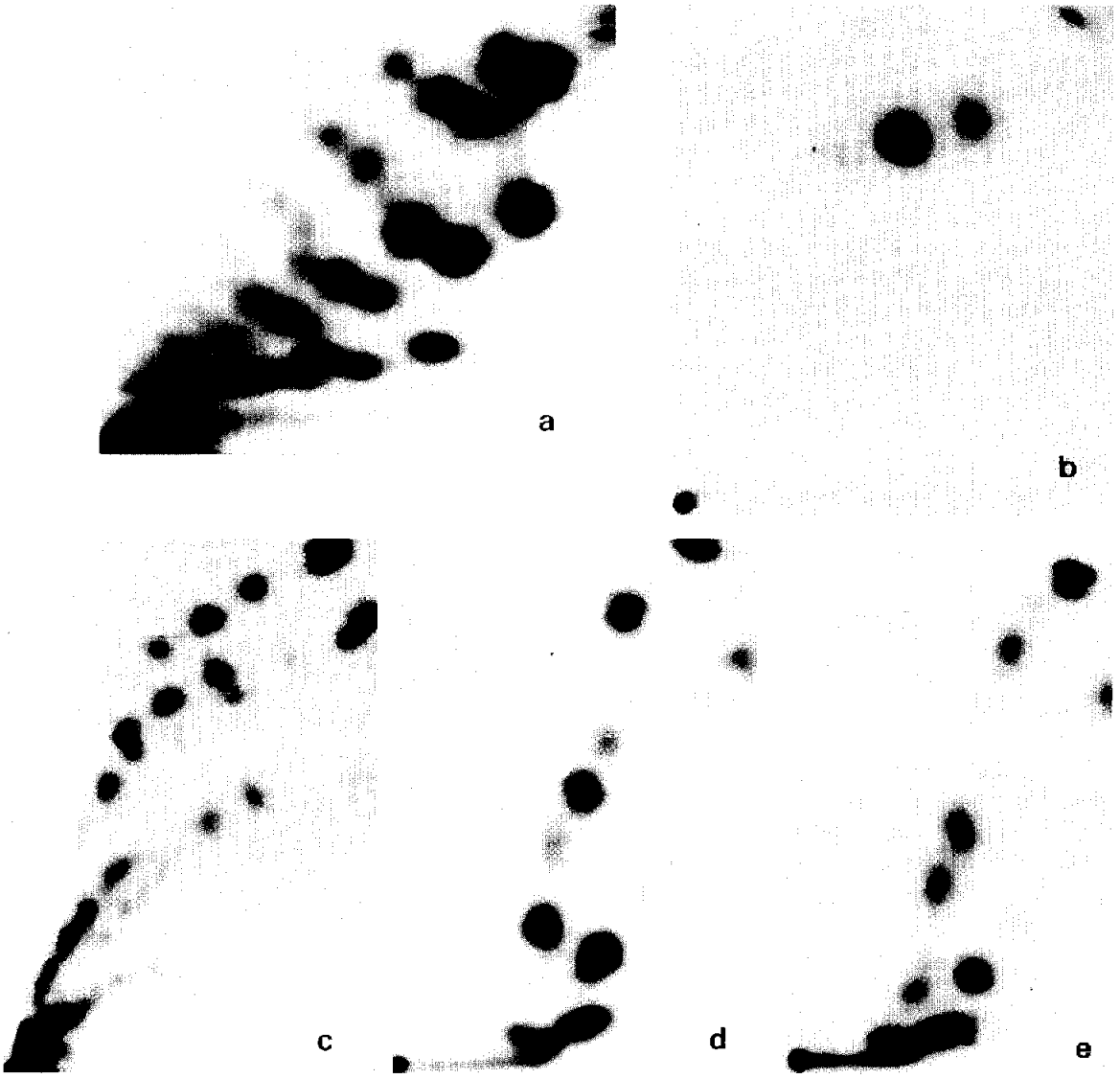

Fig.3. Two dimensional thin layer chromatography of RNase-digested RNA on PEI-cellulose. Elution of RNA from the bands I–III of fig.2, digestion and chromatography were accomplished according to Mirzabekov and Griffin [6] with the following modifications: To the precipitated RNA 20 μg tRNA was added and both were dissolved in 10 μl 10 mM Tris pH 7.8 and 2 mM Mg^{2+}. After addition of 2 μg T1-RNase (pancreatic RNase) and incubation for 30 min (10 min) at 37°C the oligonucleotides were separated: first dimension (bottom to top): 7 M urea and 1.5 M Li-formiate pH 3.4; second dimension (left to right): 7 M urea, 0.7 M LiCl and 10 mM Tris pH 8. (a) T1 RNase digestion of T7 RNA labeled with [α-^{32}P] GTP and synthesized at pH 8 (control); (b) T1 RNase digestion of [α-^{32}P] GTP labeled T7 RNA of band I (fig.2b); (c) pancreatic RNase digestion of T3 RNA labeled with [α-^{32}P] ATP and synthesized at pH 8 (control); (d) and (e) pancreatic RNase digestion of [α-^{32}P] ATP labeled T3 RNA of (d) band II and (e) band III (fig.2a).

eluted from the gel, digested by pancreatic- or T1-RNase and the products separated by two dimensional chromatography on PEI-cellulose (fig.3). The fingerprint pattern of the smallest T7 RNA (fig.3b) consisted of only 3 major spots when the RNA was labeled with [α-^{32}P] GTP and digested with T1-RNase: two spots with high mobility and one spot that hardly moved from the origin. The two patterns of T3 RNA (fig.3d and e) were more complicated than that of the small T7 RNA band (fig.3b) but considerably simpler than the control pattern of RNA synthesized at pH 8. The minor spots of these patterns were present in less than stoichoimetric amounts and may therefore derive from random RNA contaminating the bands in the slab gels.

The data shown indicate that there are only a few specific starts and stops at a few sites of the genome. Whether the starts are identical with the RNA polymerase starts at pH 8 but new terminators are operating or whether there are new initiation sites for the enzyme at low pH is not yet known. Since it is possible to get specific transcripts in large quantities, this technique may be helpful for sequence analysis of initiation sites of various RNAs.

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