SIZE OF THE RNA's SYNTHESIZED BY PURIFIED CALF THYMUS DNA-DEPENDENT RNA POLYMERASES ON SV40 DNA

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1. Introduction

Studies on the transcription *in vitro* of phage DNA's by purified *E. coli* DNA-dependent RNA-polymerase (EC 2.7.7.6) have been extremely useful in elucidating some of the mechanisms involved in the regulation of transcription in prokaryotes [1]. Similar studies using the RNA polymerases which have recently been purified from animal cells [2–4], and DNA's from animal viruses which do not contain endogenous polymerase, should throw some light upon the role of the various animal RNA polymerases in the regulation of transcription in eukaryotes. Since non-specific initiation of RNA synthesis can occur on nicked DNA's [5, 6], we decided to study the transcription *in vitro* of twisted circular double-stranded SV40 DNA Form I by purified calf thymus AI and B RNA polymerases. We report here on the lengths of the RNA's synthesized by these enzymes.

2. Materials and methods

Calf thymus RNA polymerase AI (fraction GG) was purified by a modification of the method of Gissinger and Chambon [3], and a mixture of the calf thymus B enzymes (fraction GG) was purified according to Kedinger and Chambon [4]. *E. coli* holoenzyme was prepared as previously described [7]. All enzymes were at least 95% pure and were free of DNAase. SV40 DNA Form I labeled with [^{14}C]thymidine was extracted from infected CV8 cells according to Hirt [8] and purified by isopycnic centrifugation in CsCl in the presence of ethidium bromide after phenol, and chloroform–isoamyl alcohol extraction. The preparation consisted of at least 95% twisted molecules. In some experiments the DNA was further purified by sedimentation in a neutral sucrose gradient to eliminate the multimers. Similar results were obtained with both DNA preparations. RNA was synthesized as described in the legend to the figures. In contrast to our previously published method [3], albumin was omitted from the incubation medium, since all of the commercially available preparations were contaminated with ribonuclease. The RNA's synthesized were purified by phenol extraction and ethanol precipitation and denatured by heating for 5 min at 80° in 1.1 M formaldehyde containing 5 mM sodium phosphate buffer pH 7.7 as previously described [7] before centrifugation through formaldehyde sucrose gradients as indicated in the legend to the figures. Centrifugation through 99% dimethylsulfoxide (DMSO)-sucrose gradients was performed according to Reijnders et al. [9].

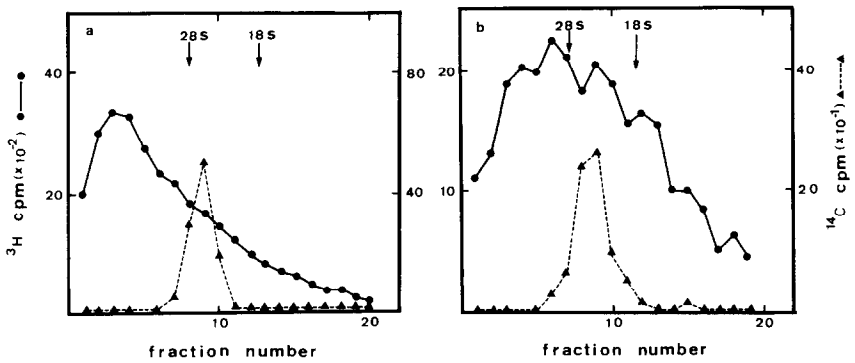

Fig. 1. Size of RNA synthesized by calf thymus enzyme AI and *E. coli* holoenzyme on SV40 DNA Form I. The incubation medium (125 μl) for RNA synthesis contained: 100 mM Tris-HCl pH 7.9, 4 mM thioglycerol, 0.1 mM EDTA, 12% glycerol, ATP, CTP and GTP, 1 mM each, and 0.12 mM [^{3}H]UTP (200–300 cpm/pmole) (Medium A). In addition the following components were added: a) 1 μg AI enzyme, 0.6 μg [^{14}C]SV40 DNA Form I, 3 mM Mn^{2+}, and 4 mM ammonium sulfate. b) 0.2 μg *E. coli* holoenzyme, 0.2 μg [^{14}C]SV40 DNA Form I, 8 mM Mg^{2+} and 120 mM KCl. RNA synthesis was started by addition of the nucleoside triphosphates after 3 min of preincubation at 37° in their absence. Incubations were stopped after 30 min for enzyme AI and 60 min for *E. coli* holoenzyme by addition of SDS (final conc. 0.5%) and processed as described in Material and methods. Centrifugations through formaldehyde–sucrose gradients (1.1 M formaldehyde, 7–27% sucrose, 0.1 M sodium phosphate buffer pH 7.7 and 1 mM EDTA) [7] were run at 28,000 rpm for 14 hr at 20° in the SW 41 Spinco rotor. About 20 fractions (0.5 ml each) were collected after centrifugation from the bottom of the tube, the acid insoluble radioactivity was collected on GF/C glass filters and counted. The arrows indicate the position of 18 S and 28 S cellular rRNA's which were used as UV markers.

3. Results and discussion

Several methods are available for determining the chain length of RNA molecules. We used the method of Boedtker [10] as modified by Fried and Sokol [7] since RNA secondary structure should be destroyed under their conditions. This was particularly necessary, since other studies [11, 12] have shown that SV40 DNA Form I is transcribed symmetrically by the animal DNA-dependent RNA polymerases. SV40 DNA Form I was incubated with calf thymus RNA polymerase AI in the presence of Mn^{2+}, since Mg^{2+} does not stimulate RNA synthesis in this system [11, 12] whereas the B enzymes were incubated with either Mn^{2+} or Mg^{2+}, since both divalent cations were found to stimulate [12].

As previously reported by Westphal [13] and Fried and Sokol [7], the RNA made by *E. coli* holoenzyme is in part larger than 28 S rRNA (fig. 1b). Most of the RNA made in 30 min by enzyme AI (fig. 1a) was also much larger than the 28 S rRNA. Since the complete transcription product of one strand of SV40 DNA would have a M.W. of about 1.6×10^6 daltons and would sediment as a 26 S RNA under these centrifugation conditions, we conclude that RNA polymerase AI can pass over its own initiation site on SV40 DNA Form I and synthesize RNA which has at least twice the length of a DNA strand. This conclusion was further supported by the results (not shown) of gradient analysis in the presence of 99% DMSO, which confirmed that the RNA's synthesized were larger than the SV40 genome.

On the contrary, the RNA's made by the calf thymus B enzymes had a definite upper size limit which was always less than a complete transcript of the genome, irrespective of the divalent cations in the medium (fig. 2a and 2b). The peak of radioactivity, which was always heterogeneous, corresponded roughly to the position of the 18 S rRNA. In fact, kinetic studies [12] have shown that chain growth stopped after 20 min. To prove that the smaller size of RNA's synthesized by the B enzymes was not due to the presence of traces of RNAase in the B enzyme preparation, we either post-incubated the RNA polymerase AI transcript with the B enzymes, or synthesized RNA with enzyme AI in the presence of the B enzymes inhibited by α-amanitin. Neither treatment altered the size of the enzyme AI products, which demonstrates that the B en-

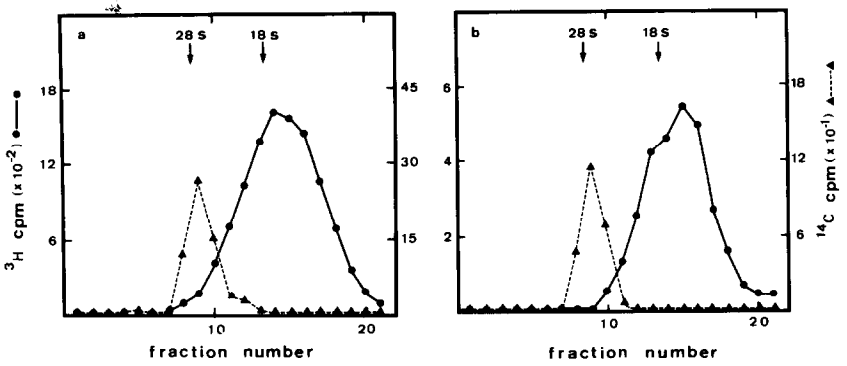

Fig. 2. Size of RNA synthesized by calf thymus enzymes B on SV40 DNA Form I in the presence of Mn^{2+} or Mg^{2+}. The incubation mixture contained: 1 μg B enzymes and 0.4 μg [^{14}C]SV40 DNA Form I in Medium A (fig. 1). Divalent cations and salt were as follows: a) 3 mM Mn^{2+}, 25 mM ammonium sulfate; b) 8 mM Mg^{2+}, 16 mM ammonium sulfate. RNA synthesis was started as described in legend to fig. 1 and stopped after 40 min at 37°. Centrifugation through formaldehyde–sucrose gradients and determination of acid-insoluble radioactivity were as in legend to fig. 1.

zymes were free of detectable RNAase. We therefore conclude that the B RNA polymerases from calf thymus (similar results were obtained with purified B enzymes from rat liver, J.L. Mandel, unpublished results) recognize some sequences on SV40 DNA which stop RNA synthesis. The size heterogeneity of the RNA's made by the B enzymes could be due either to heterogeneity of the initiation sites (unpublished results have shown that RNA chains could be initiated either by ATP or GTP) or to heterogeneity of the sites where the enzymes stop. It is interesting to note that the size of the RNA's synthesized by the B enzymes corresponds to that of the mRNA's found in infected cells [14]. Studies in progress will show whether the RNA's synthesized *in vitro* are transcribed from a defined region of the genome.

Our previous studies have shown that mammalian A and B RNA polymerases initiate at different sites on calf thymus DNA, which moreover differ from the initiation sites of the *E. coli* holoenzyme [15, 16]. Other results [11, 12] indicate that the animal enzymes transcribe SV40 DNA Form I symmetrically, whereas *E. coli* holoenzyme transcribes it asymmetrically [13]. The present results show that there are also differences between A and B enzymes at the level of RNA chain termination and support the possibility of the animal RNA polymerases playing an active role in the regulation of transcription.

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