Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 526-533

TRANSCRIPTION IN VITRO OF CAULIFLOWER MOSAIC VIRUS DNA BY RNA POLYMERASE I, II AND III PURIFIED FROM WHEAT EMBRYOS

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Received May 15, 1979

Summary. RNA polymerases I, II and III purified from wheat embryos were used to transcribe CaMV DNA in vitro. With Mn^{++} as the divalent cation the efficiencies of transcription were respectively 50%, 20% and 90% for enzymes I, II and III. With Mg^{++} only enzymes II and III could use this template; enzyme I was unable to initiate RNA synthesis. Without ATP + GTP only enzyme III could form heparin-resistant initiation complexes; in their presence heparin-resistant RNA synthesis catalyzed by enzyme III is two-fold higher than that observed with enzyme II, suggesting that the formation of highly stable initiation complexes with double-stranded DNA is an intrinsic property of enzyme III.

Introduction. At present, little is known about the mechanisms by which eukaryotic genes are activated. The presence in eukaryotic cells of three distinct classes of DNA-dependent RNA polymerases, differing in their structural properties and subcellular localization (1) suggests that transcription could be controlled, at least in part, by the use of different transcriptases to synthesize RNAs from different groups of genes.

Experiments showing that the distinct enzymes transcribe different parts of deproteinized DNA in vitro would be a conclusive argument to support this possibility. To perform such experiments purified enzymes and an appropriate DNA are required. A viral DNA can be used as template; Cauliflower Mosaic Virus (CaMV) DNA seems a convenient one. It consists of a circular double-stranded DNA molecule of 4.5 10^6 daltons (2) containing three discontinuities not randomly located (3). Furthermore, a physical map of

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this genome has been constructed on the basis of cleavage by restriction endonucleases (4,5).

On the other hand, RNA polymerases II and III from wheat embryos have been purified and characterized (6,7), and it is now possible to purify the three classes of transcriptases simultaneously (manuscript in preparation), thus, we have compared their ability to transcribe intact double-stranded DNA from CaMV. We report here our finding that RNA polymerase III transcribes this template much more efficiently than do enzymes I and II.

Materials and Methods. [^{3}H]-UTP (35 Ci/mmol) was supplied by NEN. Calf thymus DNA and heparin (160 units/mg) were obtained from Sigma. Other chemicals were purchased, heparin and tetramethylene diamine were coupled to Sepharose 4B, buffers and solutions were prepared as described (7).

Cauliflower Mosaic Virus (Cabbage B strain) obtained from Dr. P. Yot and from Dr. R.J. Shepherd was propagated in "Just Right" turnips (Brassica rapa). Virus was extracted according to Hull et al. (8), DNA was extracted and purified as described by Meagher et al. (4).

The over-all procedure for extraction and purification of RNA polymerases from wheat embryos will be published elsewhere (manuscript in preparation). It involved dissociation (3 h at 4 ° C) in 0.5 M $(NH_4)_2SO_4$, ammonium sulfate fractionation (10-50 %) and heparin-Sepharose chromatography (7), followed by hydrophobic chromatography on δ-aminobutyl-Sepharose which yielded 2 peaks of activity A and B, eluting respectively at 0.09 and 0.18 M ammonium sulfate. After concentration each peak was passed on Sepharose 4B, then chromatographed on DEAE Sephadex A-25. Peak A was resolved into enzymes I and III, peak B gave enzyme II. Enzyme I was further purified by chromatography on Carboxymethyl Sephadex C-25 (9). Each enzyme was concentrated and stored at -70° C (6).

RNA polymerase assays were conducted as described in the legends to figures and tables.

Results. Preliminary experiments were conducted to determine optimal conditions of transcription in vitro of CaMV DNA by wheat embryo RNA polymerases I, II and III. Optimal ionic strengths of 50 mM and 50-100 mM ammonium sulfate were determined for enzymes I and II. For enzyme III the rate of RNA synthesis always decreased with increasing ammonium sulfate concentration. The three enzymes showed an absolute requirement for a divalent cation, either Mn^{++} (1-1.5 mM) or Mg^{++} (5-10 mM) (not shown).

Assuming that on commercial calf thymus DNA each RNA polymerase non-specifically initiates RNA synthesis at single-stranded breaks or on denatured regions, this DNA permits an estimate, for each enzyme, of a

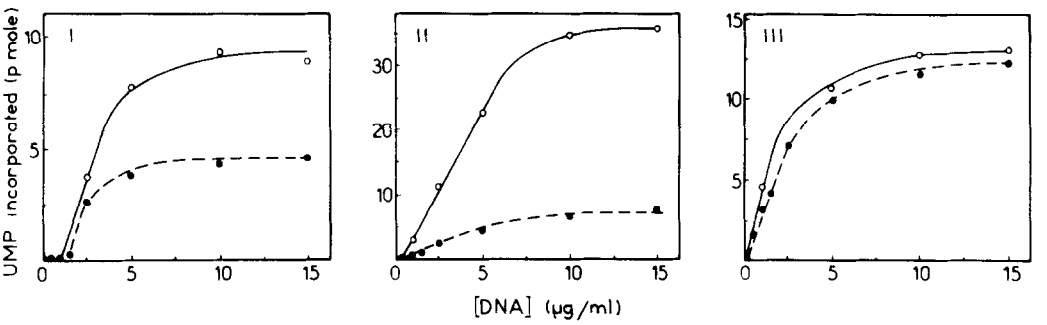

Figure 1. Transcription of CaMV DNA and commercial calf-thymus DNA by RNA polymerases I, II and III in the presence of MnSO4. The activity of a fixed amount of RNA polymerase was measured in the presence of increasing amounts of either CaMV DNA (●- - -●) or commercial calf-thymus DNA (o——o). Experimental conditions were: enzyme I 12 units per assay, 52 mM (NH4)2SO4; enzyme II 35.4 units, 60 mM (NH4)2SO4; enzyme III 20 units, 25 mM (NH4)2SO4.

Table I. Ability of wheat germ RNA polymerases I, II and III to transcribe CaMV DNA.

Enzyme	Cation	RNA synthesis with CaMV DNA pmol	RNA synthesis with calf thymus DNA pmol	Relative template efficiency %
I	Mn	6.1	12.3	49.8
I	Mg	0.2	5.6	3.6
II	Mn	11.2	57.1	19.6
II	Mg	13.4	45.2	29.7
III	Mn	14.0	15.7	89.2
III	Mg	14.2	14.5	98

Experiments were carried out with a fixed amount of RNA polymerase and a saturating amount of the indicated DNA as template, using either MnSO4 or MgCl2. The relative template efficiency is the ratio x 100 (incorporation with CaMV DNA) / (incorporation with calf-thymus DNA).

relative template efficiency of CaMV DNA. As shown in figure 1, in the presence of Mn^{++} enzyme III transcribed the viral template much more efficiently than did enzymes I or II. In the presence of Mg^{++} again enzyme III used this template more efficiently than enzyme II (Table I) whilst RNA polymerase I gave no appreciable RNA synthesis.

Heparin is known strongly to inhibit both prokaryotic and eukaryotic RNA polymerases (10, 11). This polyanion inhibits the initiation step of transcription

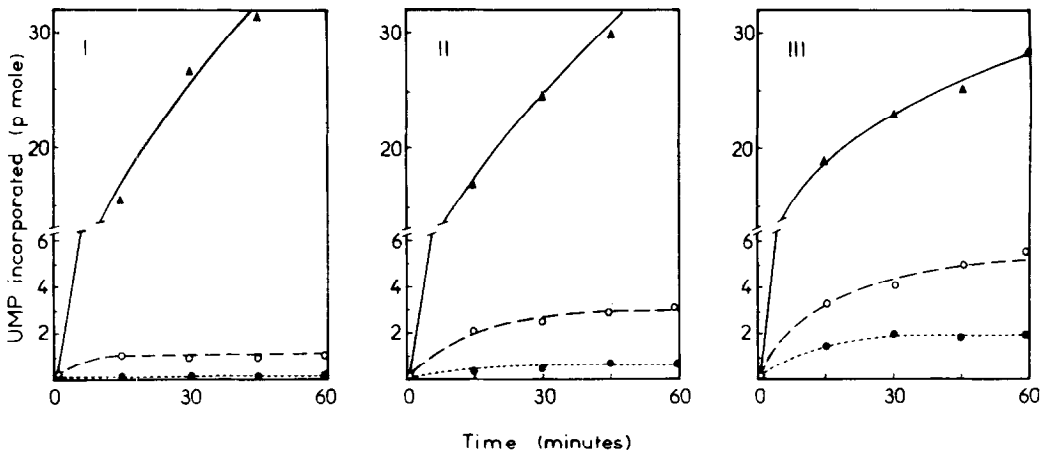

Figure 2. Time course of heparin-resistant RNA synthesis catalyzed by enzymes I, II and III transcribing CaMV DNA.
Each enzyme was preincubated for 10 minutes at 35° C with CaMV DNA in the presence of MnSO4 and either in the absence (●·····●) or in presence (o– –o) of ATP+GTP (0.5 mM each). Heparin (100 μg/ml final concentration) or an equal amount of distilled water (▲——▲) was added then the nucleoside triphosphates ATP, CTP, GTP (0.5 mM each) and [^{3}H] UTP (0.04 mM, 4μCi per assay). RNA synthesis was allowed to proceed for the indicated time. Experimental conditions were: enzyme I 1.5 mM MnSO4, 50 mM (NH4)2SO4, 0.32 μg of CaMV DNA per assay (100 μl); enzyme II: 2 mM MnSO4, 63.5mM (NH4)2SO4, 0.17 μg of CaMV DNA per assay; enzyme III: 1.5 mM MnSO4, 25 mM (NH4)2SO4, 0.32 μg of CaMV DNA per assay.

but allows elongation to occur. Thus incubating RNA polymerase with the template, then adding heparin and immediately after the four nucleoside triphosphates will allow elongation to proceed, however, under these conditions heparin prevents initiation by RNA polymerase not associated with the template in preinitiation complexes, as well as reinitiation at the end of a single round of synthesis and multiple initiations at a single initiation site. Thus, heparin-resistant RNA synthesis is a convenient means of studying enzyme·DNA complexes.

RNA polymerases I, II or III were preincubated with intact double-stranded CaMV DNA under various conditions, then heparin-resistant RNA synthesis was measured. No heparin-resistant initiation complex was observed when the preincubations were carried out at 0° C, whatever the enzyme used (results not shown), but if the preincubation took place at 35° C, RNA synthesis could be observed (Figure 2). In the absence of purine nucleoside triphosphates

ATP + GTP only enzyme III gave significant RNA synthesis, but if the preincubation step was carried out in their presence both enzymes II and III gave appreciable UMP incorporation. Moreover, under these conditions the heparin-resistant RNA synthesis catalyzed by enzyme III is higher than that observed without ATP + GTP (Table II).

In an attempt to elucidate the reason for the deficiency of RNA synthesis catalyzed by enzyme I in the presence of Mg^{++}, it seemed adequate to investigate separately the effect of either Mn^{++} or Mg^{++} on the initiation and elongation step of RNA synthesis (12). RNA synthesis with unlabeled UTP was initiated using MnSO4, 10 minutes later heparin then radioactive UTP were added and the cation concentrations were appropriately adjusted. Figure 3-A shows that either Mn^{++} or Mg^{++} allowed the elongation step, although high manganese concentrations were inhibitory. On the other hand, RNA synthesis with unlabeled UTP was performed using increasing concentrations of either MnSO4 or MgCl2, after 10 minutes heparin then radioactive UTP were added and the concentrations of Mn^{++} and Mg^{++} were adjusted to the same values. Figure 3-B shows that only manganese allowed the initiation step to occur.

Discussion. Our results indicate that the three classes of RNA polymerases from wheat germ transcribe CaMV DNA with different efficiencies. The highest relative template efficiency, estimated by comparison with commercial calf thymus DNA, was observed with enzyme III. These results agree with those previously reported indicating that the ability of class III RNA polymerase efficiently to transcribe intact duplex DNA seems an intrinsic property (13, 14). These results do not imply, of course, that CaMV DNA is transcribed in vivo by RNA polymerase III. Similarly, heparin-resistant RNA synthesis catalyzed by enzyme III is markedly higher than that observed when using enzymes II or I. It was known that stable heparin-resistant DNA binding complexes could be formed at elevated temperature by enzyme C from Xenopus ovaries (15) or from enzyme C from uninfected and Adenovirus-infected HeLa cells (16). In addition we have found that when preincubating the DNA with the enzyme at elevated temperature in the presence of magnesium enzyme III could form initiation complexes, whilst

Table II. Heparin-resistant RNA synthesis catalyzed by enzymes I, II and III transcribing CaMV DNA.

Preincubation Cation	ATP + GTP	Heparin	RNA synthesis with enzyme I pmol	%	enzyme II pmol	%	enzyme III pmol	%
Mn	-	-	16.0	100	16.9	100	18.8	100
Mn	-	+	0	0	0.4	2.4	1.4	7.4
Mn	+	+	0.9	5.6	2.1	12.4	3.3	17.6
Mg	-	-			13.5	100	16.3	100
Mg	-	+			0.05	0.4	0.95	5.8
Mg	+	+			0.4	3.1	1.6	9.7

Experiments were carried out as indicated in the legend to figure 3, except that, when indicated, 5 mM MgCl2 was used instead of MnSO4. Values reported are those obtained after 15 minutes synthesis.

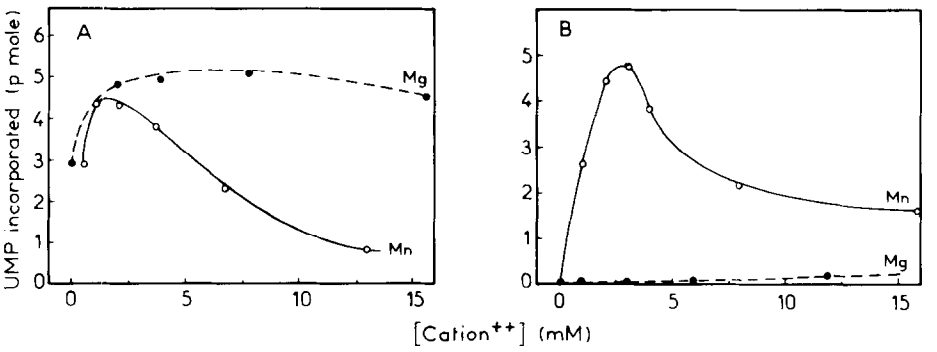

Figure 3. Effect of divalent cations on the elongation step (A) and on the initiation step (B) of RNA synthesis catalyzed by enzyme I.

A. Each assay (50 µl) contained 19.2 units of enzyme I, 1.32 µg of CaMV DNA, 50 mM (NH4)2SO4, 1.6 mM MnSO4. RNA synthesis was carried out for 10 minutes at 35° C with 0.5 mM each ATP, CTP, GTP and 0.04 mM unlabeled UTP, then heparin was added (75 µg/ml final concentration). Each assay was diluted 4-fold without changing the concentrations of any of the components except enzyme, DNA and the divalent cation either Mn^{++} (o—o) or Mg^{++} (●– –●) which was adjusted to the indicated values. 5 µCi of [3H] UTP were added and incorporation of UMP was measured 45 minutes later. The effect of Mg^{++} was examined in the presence of 0.4 mM MnSO4.

B. RNA synthesis with unlabeled UTP was carried out as indicated in A, in the presence of increasing concentrations of either MnSO4 (o—o) or MgCl2 (●– –●). Heparin was added and each assay was diluted 4-fold without changing the concentrations of any of the ingredients except enzyme, DNA and the divalent cations which were adjusted to 1.25 mM MnSO4, 5 mM MgCl2. 5 µCi of [3H] UTP were added, the incorporation of UMP was measured 45 minutes later.

enzyme I or II did not, as has been observed for mammalian RNA polymerases transcribing SV 40 DNA (17).

Hossenlopp et al. (18) showed that the nature of the divalent cation has a marked effect on RNA polymerase·DNA complex stability. They found that complexes formed at 37° C between enzyme B from calf thymus and SV 40 DNA are more stable in the presence of Mn^{++} than with Mg^{++}; in the presence of Mg^{++} the stability of complexes formed with enzyme A I is always lower. This can be compared with our finding that in the presence of Mg^{++} enzyme I is unable to initiate RNA synthesis. With enzyme II initiation is possible, but the complexes are not heparin-resistant. Thus heparin-resistant initiation complexes formed by enzyme III under these conditions could reflect a greater stability of these complexes, suggestive again of an intrinsic property of this class of enzyme.

When purine nucleoside triphosphates were included at the preinitiation stage a higher heparin-resistant RNA synthesis catalyzed by either enzyme II or enzyme III was observed. Recent data of Lescure et al. (19) demonstrate that nucleosides stabilize highly unstable complexes formed in vitro between enzyme II of calf thymus and polyoma virus DNA. Preliminary experiments showed that RNAs synthesized in vitro under standard conditions by RNA polymerase III in the presence of Mn^{++} hybridized to both strands and to all the Eco R I restriction fragments of CaMV DNA, suggesting a non-specific transcription (20). It will be of interest to study whether heparin-resistant RNA synthesis catalyzed by either enzyme II or III displays a better selectivity of transcription.

Acknowledgements. We thank Professor L. Hirth and Dr. G. Lebeurier for gift of CaMV (Cabb-S strain) DNA. The expert technical assistance of Sylvie Raymond was greatly appreciated. This work was supported in part by a grant from the CNRS (ATP chromatine).

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