TRANSCRIPTION OF CIRCULAR, SINGLE-STRANDED DNA BY MAIZE RNA POLYMERASE II

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1. Introduction

Selective initiation of RNA chains on DNA templates by eukaryotic RNA polymerases may underlie cellular differentiation [1] and responses to environmental stimuli. Initiation of RNAs with ATP and GTP by eukaryotic RNA polymerases was inferred from the incorporation of [γ-^{32}P]nucleosidetriphosphates into acid-insoluble material [2] but the products were not rigorously identified (other than viral [3]). With maize RNA polymerase II, we could demonstrate incorporation of [γ-^{32}P]ATP into products accumulated on several templates but little was DNA-dependent (unpublished). Maize polymerase selectively transcribes maize DNA rather than a heterologous DNA when offered in the same reaction mixture [4]; demonstrating a measure of selective binding and chain initiation. We turned to a homogeneous and defined DNA to determine which template properties lead to polymerase binding and RNA chain initiation. Under certain conditions, template termini are known to serve as non-specific sites of chain initiation [5]. Mandel and Chambon [6], however, have demonstrated in vitro transcription of SV40-FI DNA by calf thymus RNA polymerases A and B, indicating that unnicked templates can be transcribed by these enzymes. The use of circular ϕX174 DNA precludes termini as potential sites of initiation, unless generated by an endonuclease in the RNA polymerase preparation. In addition, nicking of ϕX174 DNA does not significantly alter the physical structure of the DNA as does nicking of super-helical SV40-FI. We report here that single-stranded circular DNA was transcribed by maize RNA polymerase II. The product accumulated as a DNA–RNA hybrid and all of the circular DNA was recovered after transcription. Nicking the circular template had no effect on the efficiency of transcription by the maize enzyme.

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2. Materials and methods

RNA polymerase II (EC 2.7.7.6) was purified from maize seedlings as described previously [7] to no less than 4 units per mg. One unit is defined as one nmole AMP incorporated per min into acid-insoluble material on calf thymus DNA at 30°C. The enzyme preparation was free of ribo- and deoxyribo-oligomers as assayed with maize polynucleotide adenylyltransferase (EC 2.7.7.19) [8] (results not shown) eliminating the possibility of endogenous oligonucleotides serving as primers for the RNA polymerase. Enzyme reaction mixtures contained 100 mM Tris-HCl (pH 8), 8 mM 2-mercaptoethanol, 1 mM each [8-^{14}C]ATP (8 Ci/mole) or ATP, [2-^{14}C]UTP (8 Ci/mole) or UTP, GTP and CTP, 0.4 mg/ml bovine serum albumin, 5 mM $MnCl_2$, and DNA and enzyme as indicated. Product formation was measured as radioactivity incorporated into trichloroacetic acid-insoluble material on filter paper disks as described previously [7] or retained by nitrocellulose filters [9].

Tritiated phage ϕX174/am3 was prepared by the procedure of Lindquist and Sinsheimer [10]. The CsCl density gradient fractions containing the phage were pooled and dialyzed against 0.05 M Tris-HCl, 0.4 NaCl, 0.02 M EDTA (pH 7.5) prior to phenol extraction. A 1.2 liter culture yielded 1.9 mg phage DNA (assuming

1 mg DNA/per ml has an absorbance of 22 at 260 nm).

Neutral, linear 5 to 20% sucrose gradients were 50 mM Tris-HCl (pH 7.5), 3 mM EDTA and 0.5 M NaCl. Alkaline, linear 5 to 20% sucrose gradients were 50 mM EDTA and 0.35 M NaOH (pH 12.4). Samples (0.5 ml) were layered on 17 ml gradients and centrifuged in a SW 27.1 rotor under conditions indicated, then fractionated by displacement with 50% sucrose and acid-insoluble radioactivity determined as above.

To prepare linear DNA, 0.65 A_{260}/ml [^{3}H]ϕX174 DNA was incubated with 2 ng/ml pancreatic DNAase I (EC 3.1.4.5) in 3 mM Tris-HCl (pH 7.2) and 10 mM $MgCl_2$ for 20 min at 30°C. The mixture was adjusted to 0.037 M EDTA, heated at 80°C for 10 min and excluded from Sephadex G100 equilibrated 5 mM Tris-HCl (pH 7.2).

3. Results and discussion

ϕX174 DNA was used by maize RNA polymerase II as a template for the incorporation of [2-^{14}C]UMP into labeled product measured as acid-insoluble material or by retention on nitrocellulose filters (fig.1). After heating, labeled product was not retained by nitrocellulose; suggesting accumulation of a DNA–RNA hybrid [11]. Utilizing carbon labeled precusors and tritiated DNA as a template, most of the reaction product cosedimented with the DNA on a neutral

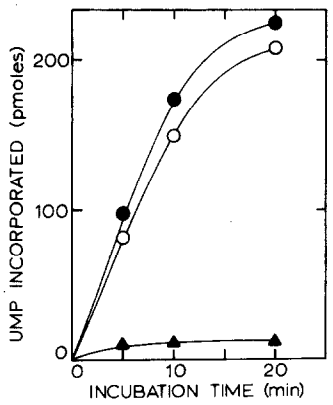

Fig.1. Accumulation of product with time. Incorporation of [2-^{14}C]UMP in a reaction mixture (0.15 ml) containing 0.2 A_{260}/ml ϕX174 DNA and 40 μg/ml enzyme into material retained by nitrocellulose filters (●-●-●), acid-insoluble material (○-○-○) and material retained on nitrocellulose filters after heating the sample at 100°C for 10 min (▲-▲-▲).

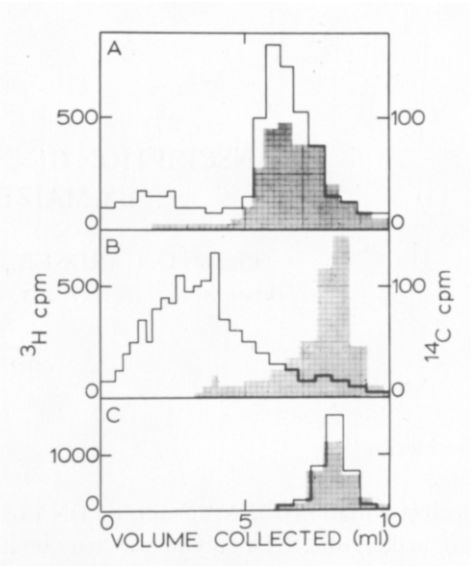

Fig.2. Neutral sucrose gradient profiles of ϕX174 DNA and polymerase products. Reaction mixtures (without albumin) were incubated at 30°C for 30 min, adjusted to 1% dodecyl sulfate, layered onto neutral sucrose gradients as described in Materials and methods and centrifuged at 20 000 rev/min for 15 h at 20°C. 15 drop fractions were collected and 0.1 ml aliquots assayed. (A) Product of a reaction mixture (0.5 ml) containing 0.08 A_{260}/ml [^{3}H] ϕX174 DNA (18 000 cpm) and 50 μg/ml enzyme and 1 mM each GTP, CTP, [8-^{14}C]ATP and [2-^{14}C]UTP. Tritium (cross-hatched). Carbon (clear). (B) Product as described in (A) was heated at 100°C for 10 min prior to centrifugation, (C) 0.08 A_{260}/ml [^{3}H]ϕX174 DNA (18 000 cpm) was incubated alone (clear) or with 50 μg/ml enzyme in a reaction mixture less nucleosidetriphosphates (cross-hatched).

sucrose gradient (fig.2A); as expected of a DNA–RNA hybrid. Since the hybrid sediments more slowly than single-stranded DNA, it is apparent that most, if not all, the DNA in a reaction mixture was transcribed (fig.2A). If the reaction product was heated prior to centrifugation, tritium labeled material sedimented like the original DNA (c.f. figs.2B with 2C) and carbon-labeled RNA sedimented more slowly (fig.2B); consistent with dissociation of a DNA–RNA hybrid. The sedimentation velocity of DNA was unchanged after incubation with enzyme (fig.2C); precluding gross contamination with nuclease.

Template DNA migrated as two bands, 14.1 S and 12.6 S (calculated per Martin and Ames [12] upon centrifugation in alkaline sucrose gradients (fig.3A); in agreement with 15 S and 13 S for circular and linear

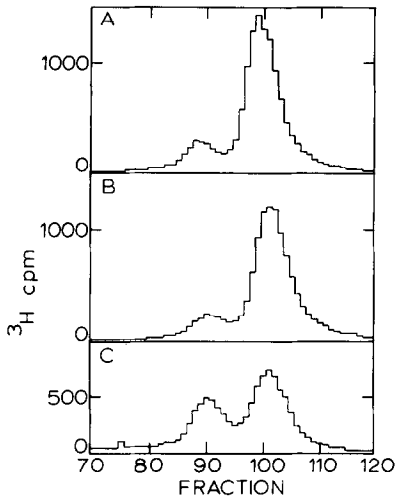

Fig.3. Alkaline sucrose gradient profiles of ϕX174 DNA. Samples (0.1 ml) were incubated at 30°C for 30 min then adjusted to 33 mM EDTA and 0.2 M NaOH (0.6 ml final vol). Samples (0.5 ml) were layered onto alkaline sucrose gradients as described Materials and methods and centrifuged at 25 000 rev/min for 30 h at 20°C. 3 drop fractions (145 per gradient) were collected directly onto filter paper disks. (A) 0.1 A_{260}/ml [^{3}H]ϕX174 DNA. (B) Reaction mixture (without albumin) containing 0.1 A_{260}/ml [^{3}H]ϕX174 DNA (16,695 cpm) and 0.015 μg/ml enzyme. (C) Reaction mixture as in B, but lacking nucleosidetriphosphates.

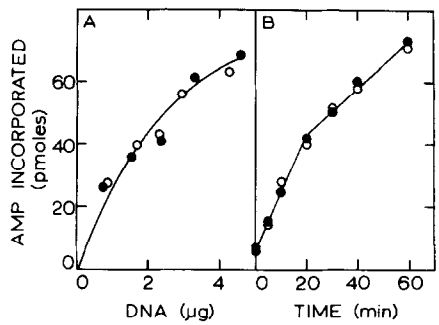

Fig.4. Efficiency of transcription of linear and circular strands. (A) Titration of maize RNA polymerase II with untreated [^{3}H]ϕX174 DNA (•-•-•) and DNAase I treated DNA (○-○-○). Reaction mixtures (0.1 ml) contained 40 μg/ml RNA polymerase. (B) Time course for the incorporation of [8-^{14}C]AMP into acid-insoluble material. Reaction mixtures (0.1 ml) contained 0.042 A_{260}/ml untreated [^{3}H]ϕX174 DNA (•-•-•) or 0.046 A_{260}/ml DNAase I treated DNA (○-○-○) and 40 μg/ml RNA polymerase.

strands, respectively, reported by Rush and Warner [13]. Prior to incubation with enzyme, the DNA was predominantly circular (79.4%) with the remaining DNA migrating as linear strands (fig.3A). DNA, incubated in a reaction mixture for 30 min, exhibited no change (fig.3B) in the proportion of circles (79.3%); indicating that circles remained intact. Omission of nucleosidetriphosphates from the reaction mixture (no product accumulated) increased the proportion of linear strands from 16.5 to 33.6% (fig.3C). Inhibition of product accumulation by α-amanitin did not enhance ring cleavage, suggesting that nucleosidetriphosphates inhibited directly the cryptic nucleolytic activity.

A template enriched in linear strands was prepared by partial digestion of ϕX174 circular DNA with DNAase I. On alkaline sucrose gradients the digested template was 31% circles, 42% rods and 27% smaller fragments. The concomitant increase in a discrete band migrating at 12.6 S with a decrease in a discrete band at 14.1 S is consistent with the nucleolytic cleavage of circles to rods [14]. Polydisperse lighter components represented subsequent cleavage of rods to smaller fragments. The amounts of labeled product accumulated on either partially digested DNA or untreated template at comparable DNA concentrations were the same (fig.4A). Similarly, the rates of AMP incorporation at limiting concentrations of either DNAase treated or untreated DNA were the same (fig.4B).

4. Conclusions

All the ϕX174 DNA present in a reaction mixture served as template for transcription by maize RNA polymerase II. The DNA was predominantly circular and no conversion to linear strands occurred with transcription. Therefore, maize RNA polymerase II, like calf thymus RNA polymerases A and B [6], initiates RNA synthesis within internal deoxynucleotide sequences. We also found that linear and circular single-stranded DNAs were transcribed with the same efficiency. It is reported that single-strand breaks in native DNA (which facilitate local denaturation) stimulate initiation by eukaryotic polymerases [5], while nicking of superhelical DNA (thus eliminating the loosely base-paired regions reported to be initiation sites) inhibits transcription [6]. These three observa-

tions suggest that in vitro initiation sites for eukaryotic RNA polymerases on all templates are locally denatured or loosely base-paired regions of the DNA, rather than nicks or ends of DNA molecules.

Acknowledgements

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