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Template Requirement of Maize RNA Polymerase[1]

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Abstract. **Maize RNA polymerase utilizes heated deoxyribonucleic acid more effectively than native deoxyribonucleic acid as a template for ribonucleic acid synthesis. A ribonucleic acid-deoxyribonucleic acid hybrid accumulates in the presence of heated deoxyribonucleic acid. The amount of product formed with either native or heat-denatured deoxyribonucleic acid does not exceed the amount of deoxyribonucleic acid added as template.**

The template requirement of several RNA polymerases (Nucleoside-triphosphate: RNA nucleotidyl transferase, EC 2.7.7.6), isolated and purified from microorganisms (5, 7, 11, 41) and mammals (13, 32), is best satisfied with native DNA. Heat denatured, calf thymus DNA actually inhibits the polymerase isolated from bovine lymphosarcoma tissue (12). In characterizing the partially purified enzyme from maize seedlings we have shown that, at relatively high levels, native or denatured DNA from several species satisfies the template requirement of the maize enzyme (42). The data presented here demonstrate that at rate limiting concentrations, denatured DNA from maize seedlings or calf thymus is 8 times more effective than native DNA in satisfying the template requirement. Furthermore, an RNA-DNA hybrid, analogous to that formed by *Azotobacter vinelandii* RNA polymerase (44), accumulates upon incubation of the enzyme with substrate and denatured DNA. The hybrid is not found when native DNA is used as template.

Materials and Methods

Enzyme Preparation and Assay. Soluble RNA polymerase was isolated from 5-day-old maize seedlings as described previously (42). The ammonium sulfate precipitate was desalted on Sephadex G-50 rather than by dialysis prior to column chromatography on diethylaminoethyl-(DEAE) cellulose. The enzyme had a specific activity of 1870. Specific activity is defined as $\mu\mu$moles of AMP incorporated in 10 minutes at 30° per milligram protein.

RNA polymerase activity was assayed as the incorporation of α-^{32}P-labeled ribonucleotides from ribonucleoside triphosphates into acid-insoluble product at 30°. A standard reaction mixture contained 40 μmoles tris-Cl (pH 8.4), 0.5 μmoles each of ATP, GTP, and UTP, 0.45 μmoles α-^{32}P-CTP (specific activity 17.4 mc/mmole), 5 μmoles magnesium acetate, 5 μmoles dithiothreitol, 20 μmoles $(NH_4)_2SO_4$, 50 μg DNA, and 50 μg of Fraction IV enzyme (42) in 0.2 ml. Samples were removed during incubation and acid-insoluble radioactivity was determined by the method of Bollum (2) as modified by Mans and Novelli (26). Products were isolated after the addition of carrier yeast RNA and $HClO_4$. Base analyses were performed on DEAE-cellulose paper by the method of Jacobson (20) as described previously (42).

Biological Materials and Reagents. Calf thymus DNA and synthetic polydeoxyribonucleotides were generously provided by F. J. Bollum. Maize DNA was prepared from 5-day-old seedlings by the method of Bolton (3), including treatment with RNase. All other materials were purchased from commercial sources. Maize grain (*Zea mays* L., WF9 X Bear 38) from Bear Hybrid Seed Company, Decatur, Illinois was germinated and the seedlings utilized for all preparations. Uniform germination and preparations of reproducible activities have been obtained with this grain for more than 5 years. Pancreatic RNase (polyribonucleotide 2-oligonucleotide-transferase, 2.7.7.16) 5 X crystallized from General Biochemicals, was heated for 10 minutes at 80° to inactivate DNase. Pancreatic DNase (deoxyribonucleate oligonucleotide-hydrolase, 3.1.4.5) electrophoretically purified and free of RNase was purchased from Worthington Biochemical Corporation. The unlabeled ribonucleotides were obtained from the following companies: P-L Biochemicals, General Biochemicals and Schwarz BioResearch, Incorporated. Labeled ribonucleotides were purchased, exclusively, from Schwarz BioResearch, Incorporated.

All experiments reported here were conducted

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with a single preparation of the appropriate DNA and individually frozen aliquots of this preparation to avoid change in DNA template activity owing to storage, salt effects or pH (21). Similarly, individually frozen aliquots of 1 RNA polymerase preparation were utilized. However, several different preparations of DNA and RNA polymerase exhibit all of the activities reported here.

Experimental Results

Template Activity of Heat Denatured DNA. The characteristics of the reaction catalyzed by maize RNA polymerase are illustrated in figure 1. The synthesis of RNA was dependent upon native or heat denatured DNA from calf thymus or maize seedlings since no incorporation of ribonucleotides was detected in the absence of DNA. The amount of RNA formed in 10 minutes incubation was a linear function of the concentration of native DNA (1–10 μg) of either species. The amount of RNA formed with heated DNA of either species was greater than that formed with native DNA. However, the concentration dependence on heated DNA was not linear. At 1 μg DNA per incubation mixture, 8 times as much RNA was synthesized with heated DNA as with native DNA. The enzyme was saturated with heated DNA at approximately 10 μg DNA at standard assay conditions. Other experiments (42) have shown that the enzyme was saturated with native calf thymus DNA at 50 μg per incubation mixture at which concentration both native and heated DNA served equally as template.

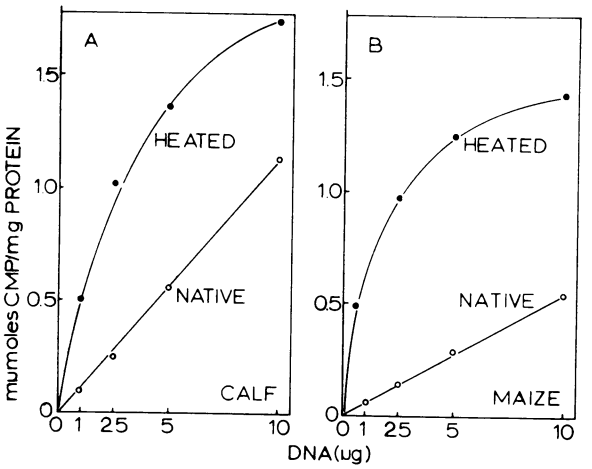

FIG. 1. Template activity of native and heat denatured DNA's. RNA polymerase activity was measured as described in Materials and Methods with the indicated amounts of calf thymus DNA (panel A) or maize seedling DNA (panel B) and incubated for 10 minutes. One $\mu\mu$mole CMP incorporated is equivalent to 31 CPM.

The incorporation of CMP by the polymerase was more efficient in the presence of low amounts of calf DNA as compared with corn DNA (fig 1). Nearest neighbor analyses of RNA's synthesized in the presence of native calf or maize DNA's (table I) indicate, but do not prove, that the product RNA's were complementary with their respective template DNA's. The fidelity of the copying process was investigated with synthetic polydeoxyribonucleotide templates. See table I. Poly dA:dT is a strand of polydeoxyriboadenylate associated with a strand of polythymidylate (24), and poly dAT is an alternating copolymer of polydeoxyriboadenylate and polythymidylate (35). The product synthesized in the presence of poly dA:dT from α-^{32}P-ATP and the other 3 ribonucleoside triphosphates contained 87.5 % of the incorporated radioactivity as ApA sequences (table I). Therefore, the major labeled product formed was polyriboadenylate. In a comparable experiment with poly dAT as the template, 99.8 % of the radioactivity incorporated from α-^{32}P-ATP was recovered as uridylic acid after alkaline hydrolysis (table I). These data strongly indicate that maize RNA polymerase copies polydeoxyribonucleotides to produce the product expected in accordance with standard Watson-Crick base pairing (6). Since the base composition of calf thymus DNA is 22 % guanine (8) and that of maize DNA is 22.8 % (10)—essentially the same—the CMP incorporated into RNA should be essentially equal. Furthermore, if we assume that, once positioned on the template, the

Table I. *Analysis of Products*

Products were formed by incubation of the indicated template in a standard reaction mixture with α-^{32}P-ATP as the labeled precursor. Alkaline hydrolysates of the acid-precipitated products were analyzed by DEAE-paper chromatography as described previously (20, 42).

Template	Total counts/min incorporated into RNA	Dinucleotide frequencies				RNA $\frac{A+U}{G+C}$	DNA $\frac{A+T}{G+C}$
		ApA	GpA	CpA	UpA		
Maize DNA	185,400	0.272	0.230	0.223	0.275	1.21	1.17[1]
Calf thymus DNA	105,150	0.308	0.228	0.208	0.255	1.30	1.29[2]
Poly dA:dT	20,700	0.875	0.030	0.028	0.069	...	...
Poly dAT	183,400	0.002	<0.001	<0.001	0.998	...	...

[1] See Ergle and Katterman (10).
[2] See Chargaff (8).

Table II. *Denaturation of DNA*

DNA	Hyperchromicity DNase[1]	Heat[2]
	%	%
Calf thymus	30.8	17.0
Maize seedling	30.0	8.3

[1] 42 μg calf thymus DNA or 46 μg maize seedling DNA was incubated for 15 minutes with 100 μg RNase free DNase in 0.1 M sodium acetate (pH 5) with 15 μmoles magnesium acetate at 30° in a final volume of 3.0 ml. The change in absorbancy at 260 nm was followed with time (23).

[2] A stoppered tube containing 42 μg calf thymus DNA or 46 μg maize seedling DNA in 1 ml SSC (28) at pH 7.4 was placed in boiling water for 10 minutes and then plunged into crushed ice. The change in absorbancy at 260 nm was measured at room temperature.

rate of polymerization for each of the ribonucleotide precursors is the same, we cannot account for the more efficient utilization of DNA observed for one species over that of another species.

The difference in template activity of the 2 DNA's is correlated with a difference in response of the DNA's to heat denaturation. Data in table II show that denaturation of DNA of either species by treatment with deoxyribonuclease (23) resulted in 30 % hyperchromicity at 260 nm. However, heating the DNA of either species in a boiling water bath followed by rapid cooling (the treatment used to prepare the heated DNA's used in the experiments of figure 1) resulted in twice the hyperchromicity (43) of calf DNA as seen with maize DNA (table II). It is not known how much of the difference in hyperchromicity between the 2 heated DNA's is a function of the separation of the 2 strands (29), the random reassociation of portions of the separated strands on cooling (9) and weakening of the internucleotide stacking forces (1). However, the greater susceptability of the calf DNA to heat denaturation does correlate with greater template activity of the calf DNA (fig 1). These data suggest that the RNA polymerase from maize requires single-stranded DNA as template. The activity seen with high levels of native DNA (42) may represent a response of the enzyme to low levels of contaminating denatured DNA or to a slow denaturation of native DNA during the incubation period.

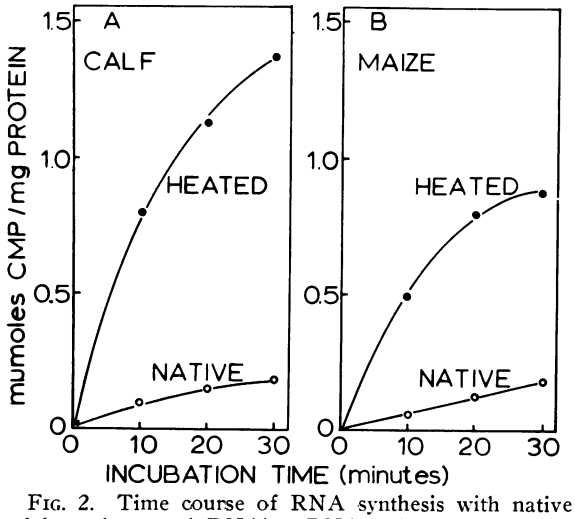

FIG. 2. Time course of RNA synthesis with native and heat denatured DNA's. RNA polymerase activity was measured as described under Materials and Methods with 1 μg of calf thymus DNA (panel A) or 1 μg maize seedling DNA (panel B). One μμmole CMP incorporated is equivalent to 31 CPM.

Rate and Amount of RNA Synthesized. The reaction rates of maize RNA polymerase on heated DNA's are significantly faster than on native DNA's. The reaction slowed appreciably in 20 minutes in the presence of heated maize DNA (fig 2B), whereas, the rate of CMP incorporation remained constant throughout the 30 minute incubation in the presence of native maize DNA. Essentially the same result was obtained with calf DNA (fig 2A). However, incorporation of CMP continued, but at a reduced rate, throughout the incubation with heated calf DNA. Calculating from the total radioactivity incorporated, the specific activity of the α-^{32}P-CTP labeled precursor used, and the base composition of the product formed in this incubation (fig 2A and table I): 0.41 μg of RNA was synthesized in the presence of 1 μg heated calf DNA in 30 minutes. Upon longer incubation, the amount of RNA synthesized did not exceed the amount of DNA template added. The slowing of the reaction rate, as the concentration of the product approached that of the heated template, suggested that an RNA-DNA hybrid (4, 14, 17, 33, 36, 38) was accumulating. The formation of an RNA-DNA hybrid would also explain the failure of pancreatic RNase to completely inhibit maize RNA polymerase when included in the reaction mixture or to completely digest the acid-insoluble product (27) since RNA-DNA hybrids are resistant to pancreatic RNase (18).

Formation of RNA-DNA Hybrid. Evidence was sought for the accumulation of an RNase resistant hybrid as a product of the maize RNA polymerase reaction. More than 90 % of the RNA synthesized with native calf or maize DNA was susceptible to digestion with pancreatic RNase (table III). However, in the presence of heat denatured DNA's more than half (73.2 % with calf and 58.6 % with maize) of the radioactive product formed in 45 minutes was resistant to RNase digestion. These results are analogous with those of Warner *et al.* (44) who demonstrated the formation of RNA-DNA hybrid with single-stranded DNA and purified RNA polymerase from *Azotobacter vinelandii*. Early in the incubation of the maize

Table III. *Hybrid Formation*

Standard 0.2 ml reaction mixtures containing 5 μg of the indicated DNA were incubated at 30° for 45 minutes. 50 μmoles of sodium acetate (pH 5), 5 μmoles EDTA and 20 μg of DNase-free RNase were added and the incubation continued for an additional 30 minutes. Changes in acid-insoluble radioactivity were followed throughout the first and second incubation periods. The data reported were calculated as:

$$\frac{\text{Total insoluble } ^{32}\text{P remaining (2nd incubation)}}{\text{Total } ^{32}\text{P incorporated (1st incubation)}} \times 100$$

Template DNA	RNase resistant product	
	Native	Denatured
	%	%
Calf thymus	8.1	73.2
Maize seedling	4.2	58.6

enzyme with denatured DNA, more than 90 % of the product was RNase resistant.

Additional evidence in support of the accumulation of an RNA-DNA hybrid in the presence of heated DNA was obtained by sucrose density gradient centrifugation of the product. The acid-insoluble, radioactive product was distributed throughout the lower two-thirds of the tube with a major component having a sedimentation coefficient of approximately 14 to 16S (fig 3A). The distribution of radioactivity correlated with the distribution of the template DNA on the gradient as indicated by absorbancy measurements at 260 nm. Essentially all the added DNA was accounted for by the absorbancy in fractions 1 through 11. The total ultraviolet absorbancy recovered from the gradient was 2.2-fold that calculated from the amount of DNA added to the gradient. Therefore, the major portion of the absorbancy exhibited in fractions 14 through 16 was ascribed to unincorporated ribonucleotides not removed from the product by dialysis. (The absorbancy data represent acid-soluble as well as acid-insoluble materials.) After the product was heated for 10 minutes at 100° and then centrifuged in the sucrose gradient, all the radioactive product was concentrated in the upper one-third of the tube peaking near the position of a 4 to 6S component (fig 3B). Similarly, the ultraviolet absorbing material of the heated product was concentrated in the upper one-third of the tube. All of the ultraviolet absorbancy observed in fractions 1 through 11 of the unheated product (fig 3A) was accounted for as the increase in absorbancy in fractions 12 through 15 of the heated product (fig 3B). The changes in the sedimentation behavior and sensitivity to RNase of the ^{32}P-labeled product after heating is consistant with the behavior of an RNA-DNA hybrid (44). Proof of hybrid formation will require physical and chemical characterization of the product isolated from an RNA polymerase incubation mixture containing heated DNA.

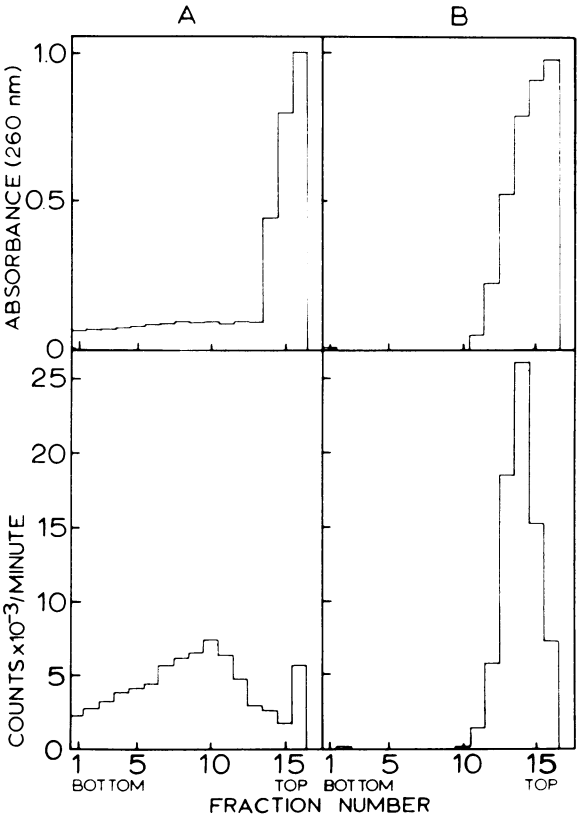

FIG. 3. Effect of heating on sedimentation of RNA polymerase product. A reaction mixture, 6-fold larger than standard, containing 300 μg of heat denatured calf thymus DNA was incubated at 30° for 30 minutes. The reaction was stopped by dilution to 0.1 M with cold sodium acetate (pH 5) and to 0.4 % sodium dodecyl sulfate. The mixture was dialyzed for 6 hours against cold, flowing 0.01 M sodium acetate (pH 5) and then against running distilled water for 18 hours at 23°. The dialyzed solution was adjusted to 0.1 × SSC (28) (pH 5), and approximately one-half (80,632 CPM) was layered over a 2.5 to 15 % sucrose gradient in 0.03 M tris-Cl (pH 7.3) and 0.1 M NaCl (panel A). The remaining portion of the dialyzed product (64,656 CPM) was heated in a boiling water bath for 10 minutes, chilled in ice and then layered as above (panel B). After centrifugation at 39,000 rpm for 3.5 hours in the SW 39 rotor at 2°, fractions were collected by dropping from the bottom of the tubes. The fractions were analyzed for acid-insoluble radioactivity and total absorbance at 260 nm. Ninety-nine percent of the radioactive products were recovered from the gradients.

Discussion

The reaction catalyzed by the RNA polymerase isolated from maize differs in several respects from the reaction catalyzed by the microbial enzymes. A) The plant enzyme uses denatured DNA more efficiently than native DNA whereas the microbial enzymes are more efficient on native DNA (5, 7,

11, 41). In its template requirement the maize RNA polymerase more nearly resembles the DNA polymerases isolated from calf thymus (2) and bacteria (34) than the microbial RNA polymerases. B) The inability of the plant enzyme to utilize RNA or synthetic oligoribonucleotide templates (42) is in contrast with their utilization by microbial enzymes (16, 22, 30, 40). C) The formation of homopolymers in the presence of heated DNA is catalyzed by the microbial enzymes (6, 40), whereas the formation of a homopolymer is not detected with the maize polymerase. D) RNA polymerases isolated from microbial cells (6, 19, 31, 39, 45) are found associated with a DNA-rich fraction of cell homogenates. The bulk of the RNA polymerase from maize seedlings is found in the DNA-poor, soluble portion of tissue homogenates (27).

These differences in template requirement between the plant and microbial enzymes are in accord with the suggestion (25) that the enzymatic apparatus required for RNA synthesis in cells having their template material (DNA) in an organized chromosome (*e.g.*, flowering plants) differs from that in cells in which the DNA is not integrated into the classical chromosomal configuration (bacteria). The more effective reversal of actinomycin D inhibition of the RNA polymerase from *Hela* cells by heat denatured DNA compared with native DNA or RNA (15), also fits into this pattern. However, data from still another tissue having organized chromosomes is not consistant with this notion. Furth and Ho (12) have demonstrated that heat denatured DNA inhibits RNA synthesis by the soluble polymerase isolated from bovine lymphosarcoma cells. Experimental verification of the hypothesis put forth by Lin *et al.* (25) must await a more extensive survey of the template requirements of RNA polymerases isolated from diverse biological species.

The inability of the maize polymerase to catalytically utilize either denatured or native templates suggests that the transcription process in this plant requires additional enzymic activities (37). Denaturation of the native template seems to be the rate limiting process. We suggest that a template-denaturing activity may be required for the catalytic use of DNA in the synthesis of plant RNA. This activity would prepare the native template for transcription by locally opening or weakening the forces holding the 2 strands of DNA together. RNA synthesis by the polymerase would then proceed along the DNA strand by way of a transient hybrid of a few nucleotides. Release of the product from the template strand may also involve an enzymic process. This final step in RNA synthesis may be catalyzed by the postulated template-denaturing activity or by still another enzymic protein. The results presented in this paper suggest that these postulated activities may have been lost in purifying the "soluble" enzyme from the DNA-poor fraction of the maize seedling homogenates (42).

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Subsequent to completion of the experimental work reported here and during the preparation of this manuscript, Karkas and Chargaff (1967) reported similar observations on the higher template activity of denatured DNA's with *E. coli* RNA polymerase. In their paper they suggest that hybrid may accumulate in the presence of low levels of denatured DNA. See Proc. Natl. Acad. Sci. 58: 1645–51.

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