DNA-Dependent RNA Polymerase from *Pseudomonas* BAL-31. II. Transcription of the Allomorphic Forms of Bacteriophage PM2 DNA†

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ABSTRACT: Transcription of the supercoiled form (I) and the relaxed circular form (II) of bacteriophage PM2 DNA was studied utilizing the DNA-dependent RNA polymerase from its host, *Pseudomonas* BAL-31. Transcription of both templates is continuous for up to 2 hr, but proceeds at a two-fold higher rate on I than on II. This difference is mainly due to a 2.2-fold higher rate of chain initiation on I. When rifampicin (Rif) is added after 10 min of synthesis, (1) transcription of II ceases by 30 min with a maximum product length of 7000 nucleotides (number average) being produced; (2) transcription of I continues with little rate reduction and with the product reaching 16,000 nucleotides (number average) by 2 hr. Sucrose gradient analysis shows that the product of II achieves maximum size 20 min after Rif addition and sediments in three peaks of 24, 33, and 39 S (approximately one-third, two-thirds, and one genome lengths). The product of I has a heterogeneous distribution and grows continuously with a large fraction reaching greater than 3 genome lengths by 90 min. The same differences in synthesis kinetics, Rif inhibition, and product size distribution are observed when I and II are transcribed by *Escherichia coli* RNA polymerase. These experiments show that (i) PM2 form I DNA is transcribed mainly by a process of continuous chain elongation, with little chain termination occurring; (ii) PM2 form II is transcribed by a process of continuous chain initiation, elongation, and termination to yield discrete products. Thus, the tertiary structure of circular DNA influences chain termination by RNA polymerase.

Previous studies on the *in vitro* transcription of bacteriophage T7 DNA have demonstrated that the DNA-dependent RNA polymerase from *Escherichia coli* is capable of recognizing and responding to initiation and termination signals in the template so as to effect the production of specific, active mRNA molecules (Millette *et al.*, 1970). This was evidenced by the production of a discrete RNA species having a unique 3′ terminus and *in vitro* messenger RNA activities for the synthesis of specific enzymes (Millette *et al.*, 1970; Schweiger *et al.*, 1971). However, since most DNA in nature occurs in the supercoiled and/or circular form in both prokaryotes and eukaryotes (Helinski and Clewell, 1971), we felt it important to extend these observations to an *in vitro* system utilizing circular duplex DNA templates.

Studies comparing the transcription of supercoiled and relaxed circular DNA forms *in vitro* have utilized mainly bacteriophage ϕX174 replicative form (Hayashi and Hayashi, 1971), SV-40 (Westphal, 1971), and bacteriophage λ (Botchan *et al.*, 1973) DNA. These studies have in general shown that there is a higher rate of transcription of the supercoiled forms (Westphal, 1971; Hayashi and Hayashi, 1971; Botchan *et al.*, 1973), only one strand is copied (Hayashi *et al.*, 1964; Westphal, 1970), and circularity is essential for asymmetric synthesis (Hayashi *et al.*, 1964). However, the effect of DNA tertiary structure on the selection of RNA initiation and termination sites is not clear. With the exception of fd RF I (Takanami *et al.*, 1971), and possibly circular λ DNA transcription (Botchan *et al.*, 1973), termination by RNA polymerase in the absence of added factors seems to be lacking on circular DNAs (Hayashi and Hayashi, 1970; Mandel and Chambon, 1974b).

In search of a model system we initiated studies on the transcription of the circular genome of bacteriophage PM2 (Espejo and Canelo, 1968a) utilizing the DNA-dependent RNA polymerase from its host, *Pseudomonas* BAL-31 (Espejo and Canelo, 1968b). With this system one can easily assess the effects of DNA tertiary structure on the transcription process, particularly on the initiation and termination phases. Moreover it has the advantage of limited genetic complexity (6×10^6 daltons of DNA, Espejo *et al.*, 1969) to facilitate product analysis.

In the preceding publication (Zimmer and Millette, 1975) we have described the purification and properties of the DNA-dependent RNA polymerase from the marine bacterium *Pseudomonas* BAL-31. With this enzyme, transcription of PM2 DNA continues for at least 2 hr, but proceeds on the supercoiled DNA (PM2-I) at a twofold higher rate than on the relaxed form (PM2-II) (Zimmer and Millette, 1975). To further investigate the transcriptional differences between these two templates, we present here a detailed study on RNA initiation, chain growth, and product size distribution using the allomorphic circular forms of PM2 DNA.

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Table I: Analysis of PM2 DNA Preparations by Electron Microscopy.

DNA Preparation	No. of Molecules Counted			% of Total		
	Form I	Form II	Form III	Form I	Form II	Form III
IA	847	172	26	81	16.5	2.5
IB	822	103	29	86	10.8	3.2
IIA	21	662	77	2.8	87.1	10.1
IIB	16	485	61	2.8	86	11.2

Materials and Methods

RNA Polymerase and DNA. DNA-dependent RNA polymerase from *Pseudomonas* BAL-31 and bacteriophage PM2 form I and form II DNAs were prepared as described in the previous paper (Zimmer and Millette, 1975). DNA-dependent RNA polymerase from *Escherichia coli* was prepared as described earlier (Millette and Trotter, 1970).

Preparation of γ-^{32}P-Labeled Ribonucleoside Triphosphates. γ-^{32}P-Labeled triphosphates were prepared by a modification of the method of Glynn and Chappell (1964). The reaction mixture was composed of 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM cysteine, 2 mM NTP, 0.01 mM 3-phosphoglycerate, 5 mCi of $^{32}P_i$, 0.2 mg of 3-phosphoglycerate kinase, and 2 mg of glyceraldehyde-3-phosphate dehydrogenase in a final volume of approximately 1 ml. The enzymes were dialyzed against 0.05 M Tris-HCl (pH 7.5)–0.006 M $MgCl_2$ for at least 1 hr before use to remove SO_4^{2-}. The mixture was incubated for 1.33 hr at 25°, 7 ml of ice cold H_2O was added, and the diluted reaction mixture was loaded onto a 1-ml QAE-A25 Sephadex column, previously washed with 0.1 N HOAc and then with H_2O to pH 6. The column was washed with 0.02 N HCl to remove $^{32}P_i$, NMP, and NDP and then with 0.2 N HCl to elute the γ-[^{32}P]NTP. This was collected, neutralized with concentrated NH_4OH, and concentrated by vacuum evaporation to about 0.2 ml. The γ-[^{32}P]NTP was desalted by passing through a Sephadex G-10 column (0.9 × 20 cm, previously equilibrated with 5 mM Tris-HCl (pH 7.9)–0.5 mM EDTA). The γ-[^{32}P]NTP was dried by vacuum evaporation and dissolved in 0.2 ml of H_2O. The final product had a specific activity of 3500–5000 cpm/pmol, contained <1% free $^{32}P_i$ as determined by PEI-cellulose thin-layer chromatography (Cashel, *et al.*, 1969), and was free of RNase as judged by the breakdown of R17 RNA (Zimmer and Millette, 1975). Recovery of $^{32}P_i$ in the form of γ-[^{32}P]NTP has ranged from 70 to 98%.

Preparation of α-^{32}P-Labeled Ribonucleoside Triphosphates. These were prepared by the procedure of Symons (1969), as modified by J. Dahlberg (personal communication), starting with 50 mCi of $H_3{}^{32}PO_4$, HCl free (New England Nuclear Corp.). The four 2′,3′-isopropylidine derivatives of adenosine (5.3 mg), guanosine (2.7 mg), cytidine (6.6 mg), and uridine (5.6 mg) (Sigma Chemical Co. or Aldrich Chemical Co.) were used together in the amounts given to yield approximately equimolar amounts of the four α-^{32}P-labeled ribonucleoside triphosphates.

Electron Microscopy of PM2 DNA. Visualization of PM2 forms I and II DNA was accomplished by using the formamide modification of the Kleinschmidt technique (Kleinschmidt and Zahn, 1959; Davis *et al.*, 1971). PM2 DNA was mixed with hyperphase buffer [40 mM Tris-HCl (pH 8.5)–4 mM EDTA] at a concentration of 1 μg/ml. Formamide and cytochrome *c* were added to final concentrations of 50% and 100 μg/ml, respectively. A 50-μl sample was then spread onto a hypophase solution prepared by mixing hypophase buffer [4 mM Tris-HCl (pH 8.5)–4 mM EDTA], H_2O, and formamide in a ratio of 3:2:1, respectively. After allowing 1 min for spreading, the DNA was picked up on freshly prepared parlodion coated grids (300 mesh), stained in a uranyl acetate solution [0.002% (w/v) uranyl acetate in 90% ethanol], shadowed with platinum/palladium at an angle of 5°, and examined in either a Philips EM200 (at 60 kV) or a Joelco JEM 100 B (at 80 kV) electron microscope. Approximately 500–1000 molecules were counted on each grid to determine the percentage of supercoiled (form I), relaxed (form II), and linear (form III) PM2 DNA molecules.

Two PM2 DNA preparations, A and B, were used in these studies. The initial distribution on the ethidium bromide–cesium chloride density gradients showed these preparations to be composed of 30 and 40% form I DNA, respectively, indicating an average of approximately one single-strand break per DNA molecule. After the dye-buoyant density purification, electron microscopic examination gave the composition for the two resulting form I and form II DNA preparations as shown in Table I.

Transcription of PM2 DNA. RNA synthesis was carried out at 28° in a "standard transcription mixture" composed of 30 mM Tris-HCl (pH 8.3), 30 mM $MgCl_2$, 150 mM KCl, and 0.5 mM each of ATP, CTP, GTP, and UTP. Reaction volumes, RNA polymerase and DNA concentrations, and radioactively labeled substrates used are indicated in figure and table legends. Incorporation of labeled nucleotides into RNA was assayed as described in the preceding paper (Zimmer and Millette, 1975).

RNA Chain Initiation Assay. The reaction mixtures were as described for standard transcription mixtures (previous section) except that either γ-[^{32}P]ATP or γ-[^{32}P]GTP (specific activities given in legends) and [^{3}H]UTP (2840 cpm/nmol) were substituted for the respective unlabeled triphosphates. Aliquots of 10 μl were taken at various times, diluted into 0.5 ml of H_2O (0°) containing 5 μmol of unlabeled ATP. The samples were precipitated with 0.5 ml of cold 10% Cl_3CCOOH and collected on Whatman (GF/C) glass fiber filters by suction filtration. Each filter was washed 10 times with about 10 ml of ice-cold 5% Cl_3CCOOH. The tops of the filtering apparatus were then removed so that the edges of the filters could be carefully washed (10 times with 1–2 ml of ice-cold 5% Cl_3CCOOH) to remove unincorporated γ-[^{32}P]NTP. After final washing four times with 1–2 ml of cold 95% ethanol, the filters were dried and incorporated radioactivity determined by liquid scintillation spectroscopy.

Sucrose Density Gradient Centrifugation. Aliquots of RNA synthesis reaction mixtures (10–25 μl) were incubated 10 min at 37° with 20 μg/ml of "RNase-free" DNase I (Worthington). The DNase was pretreated with iodoacetate to remove residual RNase activity (Laskowski, 1966). Then an equal volume of 0.3 M EDTA (pH 7.5) and H_2O were added to 0.1 ml. Five microliters of 10% sodium dodecyl sulfate (SDS) and 0.15 ml of heating buffer [10 mM Tris (pH 7.5)– 1 mM EDTA–0.5% (w/v) SDS] were added. The samples were heated at 85° for 5 min, chilled on ice, layered onto 12.5-ml, linear 5–30% (w/v) sucrose density

Table II: 5′-Nucleoside Triphosphate Initiation on PM2 DNA with *Pseudomonas* BAL-31 RNA Polymerase.[a]

DNA	γ-[^{32}P]-Ribonucleoside Triphosphate	pmol Incorp. Expt 1	pmol Incorp. Expt 2	% of Total Initiation
PM2 I	ATP	2.78	3.30	43.0
	GTP	3.13	3.85	50.0
	CTP	0.02	0.19	1.5
	UTP	0.38	0.40	5.5
PM2 II	ATP	1.19	1.23	30.5
	GTP	2.50	2.29	60.5
	CTP	0.04	0.08	1.5
	UTP	0.43	0.16	7.5

[a] Each tube contained the standard transcription mixture (50 μl) with 2.5 μg of DNA and 7.0 μg of *Pseudomonas* BAL-31 RNA polymerase in 50 μl. Incubation was for 30 min at 28°. The entire reaction mixture was precipitated, washed, and assayed for radioactivity as described under Materials and Methods. The specific activities of the γ-[^{32}P]NTP's ranged from 340 to 512 cpm/pmol.

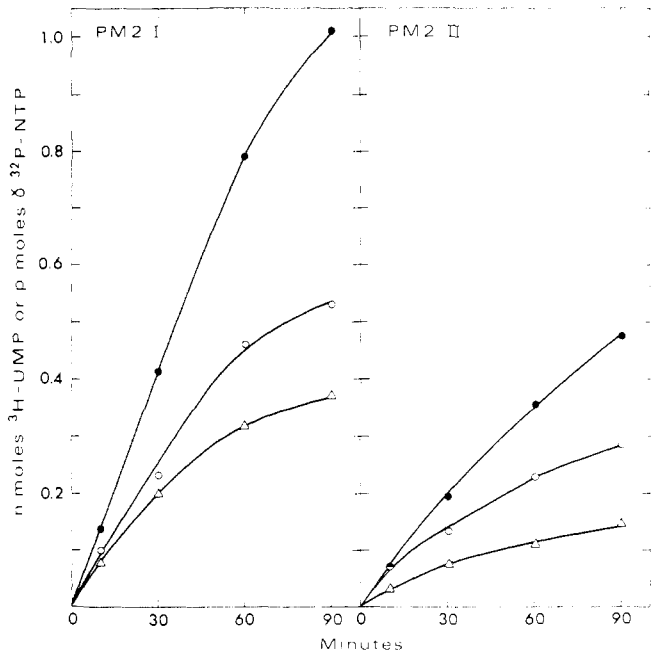

FIGURE 1: Kinetics of RNA initiation and synthesis on PM2 I and PM2 II DNA. The experimental procedure and reaction conditions were as described under Materials and Methods. Each reaction mixture contained 4.5 μg of PM2 I or PM2 II DNA, 3.2 μg of *Pseudomonas* BAL-31 RNA polymerase and either γ[^{32}P]ATP (1980–4560 cpm/pmol) or γ-[^{32}P]GTP (1055–2910 cpm/pmol): (●) [^{3}H]UTP; (○) γ-[^{32}P]GTP; (△) γ-[^{32}P]ATP.

gradients in NET-SDS [0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5% (w/v) SDS], and centrifuged at 37,000 rpm in a SW41 rotor for 3.25 hr at 20°. A total of 32–40 fractions were collected from each gradient. Following the addition of 40 μg of bovine serum albumin to each fraction as carrier, the fractions were precipitated by the addition of 1.0 ml of cold 10% (w/v) Cl_3CCOOH, and the samples were filtered and assayed for radioactivity as previously described (Zimmer and Millette, 1975).

Determination of Sedimentation Coefficients. Sedimentation coefficients of the transcription products were determined from their position in sucrose gradients relative to RNA or DNA of known *s* value, assuming constant sedimentation velocity throughout the gradients. Standards used for this purpose were ^{3}H- or ^{14}C-labeled T4 DNA (61 S), ^{14}C-labeled T7 DNA (32 S), and ^{3}H-labeled 18S RNA. Standards labeled with ^{14}C were usually run in parallel gradients, but at least one sample gradient contained ^{3}H-labeled T4 DNA and/or 18S RNA as an internal control.

Results

Initiation of RNA Chains on PM2 I and PM 2 II DNAs. In the preceding publication we reported that the *Pseudomonas* BAL-31 RNA polymerase exhibits greater activity on superhelical PM2 DNA (form I) than on the relaxed circular PM2 DNA (form II). With both templates transcription continues with little reduction in rate for at least 90 min of synthesis, while both the initial rate and total amount of RNA synthesized are about twofold greater with PM2 I DNA as template. This difference could be due to such factors as a lower initiation rate on PM2 II, higher chain growth rate on PM2 I, and/or less frequent RNA chain termination on PM2 I DNA.

To assess the role of initiation in the observed template differences, we have investigated the frequency of initiation by the four ribonucleoside triphosphates with PM2 I and PM2 II DNA (Table II). As is the case with all polymerases studied thus far, *Pseudomonas* BAL-31 polymerase initiates RNA chains predominantly with purine nucleoside triphosphates. The percentage of purine initiation is 93% on PM2 I and 91% on PM2 II DNA. There is negligible initiation with cytosine triphosphate and only about 5–8% with uridine triphosphate.

With PM2 I DNA, the relative number of chains initiated, as judged by the total γ-[^{32}P]NTP incorporation, is about twice that seen with PM2 II. This agrees with the observed differences in template activities of the two DNAs. Furthermore, with PM2 I DNA there is a proportionally higher frequency of initiation with ATP. The ratio of ATP to GTP initiation with PM2 I DNA is 0.86 while with PM2 II it is 0.50.

The difference in ATP and GTP initiation on the two circular forms of PM2 DNA was studied in more detail by an examination of the kinetics of initiation on the two DNA templates (Figure 1). With both templates initiation continues throughout the 90-min period. These results again illustrate the higher initiation rate on form I DNA. After 60 min of synthesis the number of RNA chains initiated on PM2 I DNA is 2.36-fold greater than on PM2 II DNA; after 90 min it is 2.11-fold greater. Moreover, the higher ratio of ATP to GTP initiation with PM2 I DNA holds throughout the entire time course of synthesis. From an average of the individual time points we find that the per cent initiation with ATP (disregarding CTP or UTP initiations) is 44% with the form I and 33% with form II DNA.

By normalizing RNA synthesis on the two templates we find that the relative number of chains which initiate with GTP is approximately the same for both DNAs whereas there are only two-thirds as many ATP initiations on PM2 II as on PM2 I DNA. This could be due to differences in the number of ATP initiation sites and/or the rate of initiation at ATP sites.

Effect of Rifampicin on the Kinetics of PM2 DNA Transcription. We have shown that both forms of PM2 DNA

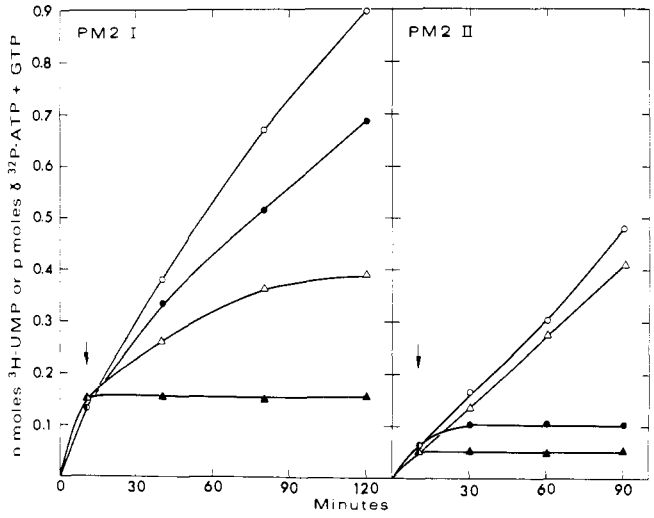

FIGURE 2: Effect of rifampicin on RNA initiation and synthesis with PM2 I and PM2 II DNA. Two 60-μl "standard transcription mixtures" containing [^{3}H]UTP (2.84 cpm/pmol) and γ-[^{32}P]GTP (2410 cpm/pmol) were prepared. To one tube was added 6 μg of PM2 I DNA and 1.8 μg of *Pseudomonas* BAL-31 RNA polymerase. To the other tube was added 3.6 μg of PM2 II DNA + 2.7 μg of polymerase. Rifampicin (20 μg/ml) was added 10 min after the start of RNA synthesis (arrows). Experimental procedure was as described under Materials and Methods: (○) [^{3}H]UMP in the absence of rifampicin; (●) [^{3}H]UMP in the presence of rifampicin; (△) γ-[^{32}P]GTP in the absence of rifampicin; (▲) γ-[^{32}P]GTP in the presence of rifampicin.

support continuous synthesis of RNA at least over a period of 90 min. This could be the result of continuous elongation of nascent RNA chains and/or continuous RNA initiation, synthesis, and termination by the polymerase. In order to distinguish between these possibilities the antibiotic rifampicin was used. We have previously shown that *Pseudomonas* BAL-31 RNA polymerase is completely inhibited by rifampicin at 4×10^{-6} M (Zimmer and Millette, 1975). Assuming that rifampicin inhibits *Pseudomonas* BAL-31 polymerase in the same manner as it does *E. coli* polymerase, it should block chain initiation (Sippel and Hartman, 1968). If this is the case, addition of the drug at some time after the start of synthesis should rapidly stop transcription if continuous initiation and termination of synthesis by RNA polymerase were taking place. Alternatively, if synthesis were mainly due to continued chain extension, rifampicin should have little effect on the synthesis kinetics. The results of such an experiment, where rifampicin is added 10 min after the start of RNA synthesis with PM2 I and II DNA, are shown in Figure 2. As measured by the incorporation of γ-[^{32}P]GTP, rifampicin immediately blocks chain initiation by *Pseudomonas* BAL-31 polymerase on both templates. With PM2 II DNA the incorporation of [^{3}H]UMP ceases within 20 min after rifampicin addition. However, with PM2 I DNA it has little effect on the overall kinetics of RNA synthesis. Thus, with PM2 I DNA continuous synthesis appears to involve mainly the extension of nascent RNA chains, while with PM2 II DNA continuous transcription requires continuous initiation by RNA polymerase. Further support for RNA synthesis occurring mainly *via* chain elongation on PM2 I DNA comes from the kinetics of initiation in the absence of rifampicin. If enzyme molecules were not being recycled, the concentration of free polymerase and, consequently, the rate of chain initiation should continuously decrease. The experimental conditions for PM2 I DNA transcription (low enzyme/DNA ratio, long incubation time) were chosen to demonstrate this more clearly. As shown in Figure 2, the rate of initiation on PM2 I DNA decreases throughout the time course and essentially stops by 120 min.

Table III: Number Average Chain Length of RNA Synthesized on PM2 Form I and Form II DNA.[a]

PM2 I			PM2 II		
Min	−Rif	+Rif	Min	−Rif	+Rif
10	3170	2,980	10	3500	4230
40	4900	7,550	30	4310	6900
80	6490	11,710	60	3920	7090
120	8040	15,670	90	4020	6950

[a] Experimental procedures are described in Figure 2. The number average chain lengths are calculated as described under Materials and Methods from the data in Figure 2.

Chain Lengths of PM2 DNA Transcription Products. From the data presented in Figure 2, the number average chain lengths of the RNA molecules formed by transcription of PM2 I and PM2 II DNA have been calculated (Table III). With PM2 I DNA, in the absence of rifampicin, the chain length increases from 3170 nucleotides at 10 min to 8040 at 2 hr of synthesis. This is slightly less than one complete genome copy which would be approximately 9500 nucleotides in length. The data obtained after the addition of rifampicin to block the continuous input of newly initiated chains give a truer picture of the final size of the RNA product. In this case the RNA chains grow from a number average length of 2980 at 10 min to 15,670 nucleotides at 120 min, corresponding in length to 1.65 copies of the genome. This is again consistent with a transcription mechanism involving continuous RNA chain extension on PM2 I DNA.

With PM2 II DNA the number average chain length of RNA synthesized in the absence of rifampicin reaches a maximum of about 4000 nucleotides by 30 min and thereafter does not appreciably change throughout the course of RNA synthesis (Table III). In the presence of rifampicin, where nascent RNA chains are allowed to be completed, the average chain length reaches a maximum size in 30 min of 7000 nucleotides or about 70% of one complete genome copy. This "plateauing" of the average product chain length, in both the presence and absence of rifampicin, supports a mechanism of continuous initiation and termination by RNA polymerase on the form II DNA.

Sucrose Gradient Analysis of PM2 Transcription Products. The preceding chain length analysis of RNA products indicates that PM2 II DNA yields products of limited terminal size whereas PM2 I products continue to grow to sizes greater than one genome copy. However, these observations do not rule out discrete product formation from both templates by post-transcriptional cleavage (Dunn and Studier, 1973) or termination by a factor such as ρ (Roberts, 1969). Preliminary analysis of the 60-min RNA products by SDS-sucrose gradient sedimentation showed the PM2 I product to have a heterogeneous distribution from 10 to 65 S with a peak around 33 S. The PM2 II product, however, was found to consist of three discrete peaks of about 26, 32, and 38 S (data not shown).

In order to obtain a clearer picture of RNA product for-

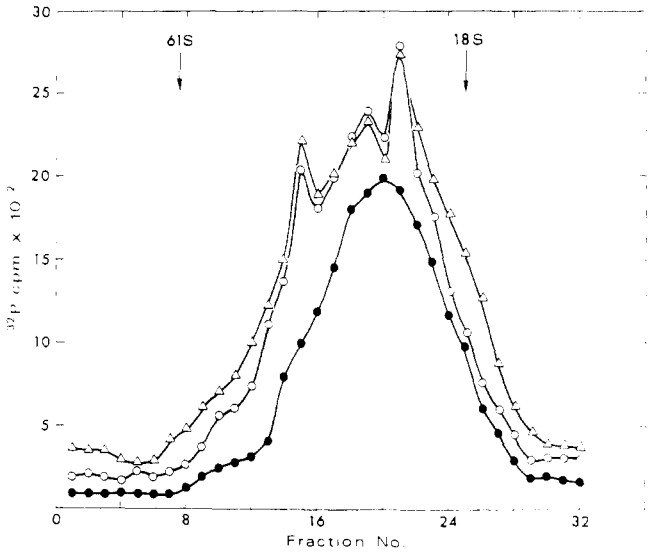

FIGURE 3: RNA transcription products of PM2 II DNA. An 80-μl standard transcription mixture utilizing α-[^{32}P]NTP (25 cpm/pmol) as substrate and containing 4.8 μg of PM2 II DNA and 4.2 μg of RNA polymerase was prepared. Rifampicin (20 μg/ml) was added at 20 min of synthesis. Twenty-microliter samples were removed at 20 (27,000 cpm), 40 (38,000 cpm), and 60 (40,000 cpm) min, and analyzed by sucrose gradient centrifugation as described under Materials and Methods: (●) RNA at 20 min; (○) RNA at 40 min; (△) RNA at 60 min of synthesis.

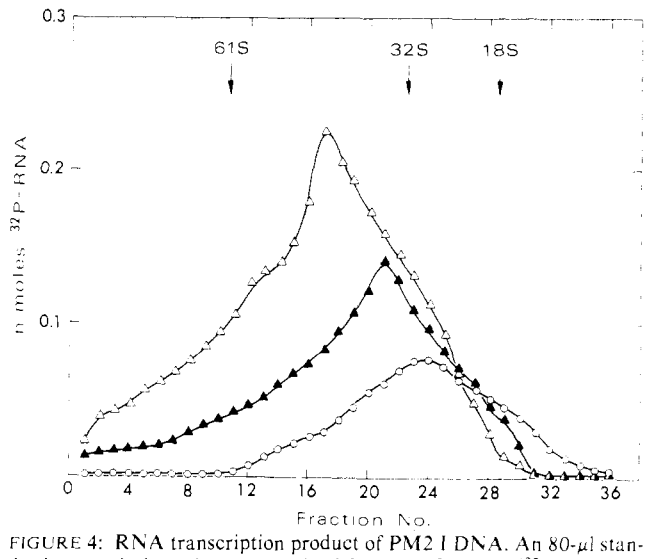

FIGURE 4: RNA transcription product of PM2 I DNA. An 80-μl standard transcription mixture contained 0.5 mM of each α-[^{32}P]NTP (20 cpm/pmol), 6 μg of DNA, and 2.7 μg of *Pseudomonas* BAL-31 polymerase. Twenty-five microliter samples were removed at 20, 60, and 90 min and analyzed by SDS-sucrose density gradient analysis as described under Materials and Methods. Rifampicin (20 μg/ml) was added at 20 min: (○) 20 min; (▲) 40 min; (△) 90 min of synthesis.

Table IV: Sedimentation Coefficients and Molecular Weights of RNA Transcribed from PM2 II DNA.

Sucrose Gradient Peaks	Sedimentation Coeff	Mol Wt × 10^{-6} [a]
I	24.3 ± 0.8	1.25 ± 0.18
II	32.7 ± 1.3	2.35 ± 0.22
III	39.3 ± 1.3	3.44 ± 0.21

[a] $M = 1550S^{2.1}$ (Spirin, 1961).

mation from the two templates it was again necessary to block the continuous input of newly initiated chains with rifampicin in order to follow the growth of nascent RNA chains. The products of PM2 II transcription synthesized after addition of rifampicin at 20 min are shown in Figure 3. At 20 min after the onset of RNA synthesis the RNA product is heterodisperse, with an average size around 30 S. By 40 min (20 min after rifampicin addition) three peaks of RNA appear. The sedimentation coefficients of these peaks, 27, 33, and 42 S, are similar to those observed after 60 min of synthesis in the absence of rifampicin. The RNA profile remains essentially unchanged from 40 to 60 min of synthesis.

From six separate sedimentation analyses of the PM2 II transcription product the average sedimentation coefficients for the three RNA peaks, and their respective molecular weights, were calculated (Table IV). Since one complete copy of the PM2 genome would produce an RNA molecule of approximately 3.2 × 10^6 daltons, the molecular weights for these peaks correspond to roughly one-third, two-thirds, and one genome copy.

Sedimentation analysis of the transcription product from PM2 I DNA is shown in Figure 4. In this case the product continues to increase in size throughout the time course with no evidence of discrete product formation. The RNA profiles remain heterogeneous and grow from an average value of 28 S at 20 min to 36 S at 60 min. The 90-min RNA peak has a sedimentation value of 45 S which is significantly larger than one complete genome copy. Moreover, an appreciable portion of this RNA has a sedimentation coefficient of greater than 61 S, corresponding to a molecular weight of greater than 8.6 × 10^6 daltons as calculated from Spirin's RNA relationship (Spirin, 1961). RNA of this size would be equal to or greater than three genome equivalents in length. Thus the RNA polymerase does not seem to be able to recognize termination sites on PM2 I DNA and continues to transcribe it through several rounds.

Growth Rate of RNA Chains on PM2 DNA. The number average chain growth rates on both forms of PM2 DNA can be calculated from the double labeling experiments, and from the size of the products as determined by sucrose gradient zone sedimentation. From the number average chain lengths at 10 min of synthesis (Figures 1 and 2) average growth rates of 5.1 nucleotides/sec with PM2 I and 6.3 nucleotides/sec with PM2 II DNA are obtained.

From the average s values for the 20-min transcription products on sucrose density gradients, average growth rates of 4.3 and 5.7 nucleotides/sec are obtained for PM2 I and PM2 II directed RNA synthesis, respectively. Thus, the higher rate of RNA synthesis on PM2 I DNA cannot be explained by a faster chain growth rate. In fact, the data show the chain growth rate on PM2 II to be slightly faster than that on PM2 I DNA.

Transcription of PM2 DNA by E. coli Polymerase. To test if the observed differences in the transcription of PM2 I and II DNA are mainly a function of the template or of the enzyme, we examined the kinetics of transcription of PM2 I and PM2 II DNA by *E. coli* polymerase in the presence and absence of rifampicin (Figure 5). As is the case with *Pseudomonas* BAL-31 polymerase, the rate of RNA synthesis on PM2 I DNA is approximately twofold greater than that on PM2 II DNA. In addition, transcription is shut off by rifampicin only when PM2 II DNA is the tem-

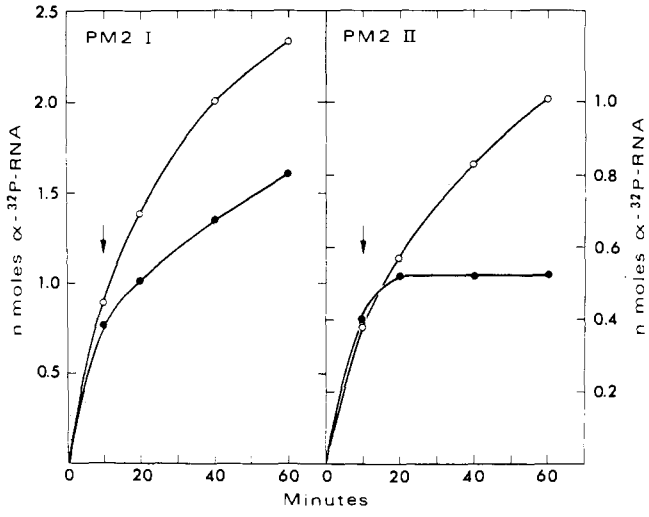

FIGURE 5: Transcription of PM2 I and PM2 II DNA by *E. coli* polymerase. Each 50-μl standard transcription mixture contained 0.5 mM of each α-[^{32}P]NTP (40 cpm/pmol), 2.5 μg of either PM2 I or PM2 II DNA, and 3 μg of *E. coli* polymerase. After 10 min of incubation at 28°, 1 μg of rifampicin was added to each reaction. Aliquots (10 μl) were removed at the indicated times, precipitated with Cl_3CCOOH, and assayed for radioactivity as described under Materials and Methods: (○) no rifampicin added; (●) rifampicin added at 10 min.

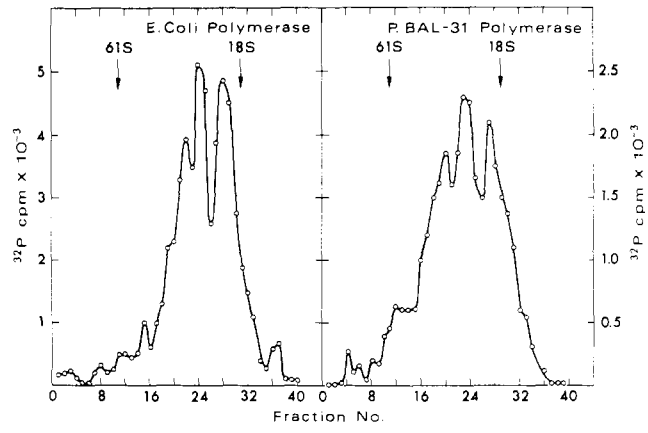

FIGURE 6: Products of PM2 II DNA transcription by *E. coli* and *Pseudomonas* BAL-31 RNA polymerase. The 50-μl standard transcription mixtures contained 0.5 mM of each α-[^{32}P]NTP (100 cpm/pmol), 2.5 μg of PM2 II DNA, and 6 μg of either *E. coli* or *Pseudomonas* BAL-31 polymerase. After a 45-min incubation 10-μl samples were removed and analyzed as described under Materials and Methods. The sample from the *E. coli* reaction mixture contained 162,000 cpm of RNA, and the *Pseudomonas* BAL-31 sample contained 55,000 cpm of RNA.

plate. The similarity of these results to those obtained with *Pseudomonas* BAL-31 polymerase suggests that termination of transcription on PM2 DNA depends on its tertiary structure.

When the products of transcription of PM2 II DNA by *E. coli* polymerase are analyzed on SDS-sucrose density gradients, three main RNA peaks with sedimentation coefficients of 25, 34, and 38 S are found (Figure 6). These agree closely with the average sedimentation coefficients of 24, 33, and 39 S, calculated for the products of *Pseudomonas* BAL-31 polymerase transcription in the same analysis.

Discussion

The foregoing studies with RNA polymerase from *Pseudomonas* BAL-31 have shown that bacteriophage PM2 I DNA, the supercoiled circular form, is a more active template for RNA synthesis than PM2 II DNA, the relaxed circular form. We have observed that both the rate of RNA synthesis and the rate of chain initiation on PM2 I DNA are about twofold higher than the rate on PM2 II DNA. There are a number of possible factors which could contribute to these differences. (1) Form I DNA might permit a higher growth rate of RNA chains. This can be ruled out since we have calculated that the average chain elongation rate is approximately 1.2-fold higher on PM2 II than on PM2 I. (2) A higher frequency of chain termination on form II could lower the overall rate of transcription by increasing the fraction of polymerase molecules in the recycling phase between termination and reinitiation. This cannot explain the observed differences since the rate of transcription of PM2 II even during the first 10 min is only one-half that of PM2 I. From the average 10-min chain growth rate of 6 nucleotides/sec, one can calculate that only a small fraction of the polymerase molecules which initiate could reach the first termination site (specifying the 24S product) within 10 min. Moreover, if termination were a major contributing factor, then the rates of initiation would initially be similar; clearly this is not the case. (3) Single-strand breaks in the PM2 II DNA could serve as nonproductive enzyme binding or initiation sites, reducing the free enzyme concentration and thus the overall initiation rate. It has been shown that an average of one *E. coli* holoenzyme molecule binds to one single-strand break in T7 DNA (Dausse *et al.*, 1972; Hinkle *et al.*, 1972). But since the form II DNA we have used was derived from DNA which contained an average of one single-strand scission per molecule and since these experiments were run at a molar enzyme to DNA ratio of 9, it is unlikely that the effective enzyme concentration would be sufficiently reduced to give the observed differences. (4) PM2 I DNA provides either a greater number or more efficient initiation sites for the enzyme. Since the rate of chain elongation is approximately the same for both templates, the initial rate of transcription should be proportional to the number on polymerase molecules which have initiated synthesis. The data of Figure 1 show this to be true: at 10 min, the rate of synthesis is 2.0-fold higher and the rate of initiation is 1.8-fold higher on PM2 I DNA.

What is the reason for the higher initiation rate with form I DNA? It could be due to a greater number of available initiation sites and/or to a higher rate of initiation at the same number of sites, possibly caused by a higher binding affinity of the enzyme for the form I DNA. Although our data do not allow us to distinguish between these possibilities, observations on the transcription of other circular DNAs may help to interpret our findings. The main studies to date comparing the transcription of superhelical (form I) *vs.* relaxed circular (form II) or linear (form III) DNA have been with SV-40 DNA (Westphal, 1971; Hossenlopp *et al.*, 1974; Mandel and Chambon, 1974b), ϕX-174 replicative forms (Hayashi and Hayashi, 1971), and circular λ DNA (Botchan *et al.*, 1973). There is general agreement among these studies in that (i) superhelical DNA supports a twofold or greater rate of transcription than the relaxed form; (ii) this difference is presumably due to a higher rate of initiation (Botchan *et al.*, 1973); (iii) the higher rate of transcription of superhelical DNA can be attributed to a more stable polymerase-DNA binding as measured by rifampicin-resistant complex formation (Westphal, 1971; Hayashi and Hayashi, 1971) or by retention of the enzyme-

DNA complex on nitrocellulose filters (Hossenlopp *et al.*, 1974).

Tighter binding of the enzyme to form I DNA could be favored by a partial unwinding of the supercoiled DNA by RNA polymerase and/or by the presence of non-hydrogen-bonded regions in the DNA. Saucier and Wang (1972) showed that the binding of RNA polymerase to supercoiled DNA causes a slight unwinding of the DNA helix of about four base pairs or 120 deg. This causes an increased binding affinity of polymerase to DNA with increasing negative superhelicity (Botchan *et al.*, 1973). The existence of unpaired or weakly hydrogen-bonded regions in PM2 I and other superhelical DNAs has been demonstrated by reactivity with methylmercuric hydroxide (Beerman and Lebowitz, 1973) and formaldehyde (Dean and Lebowitz, 1971; Jacob *et al.*, 1974), and susceptibility to single-strand specific nucleases (Kato *et al.*, 1973; Beard *et al.*, 1973). That such regions in supercoiled DNA may serve as polymerase binding sites is shown by the observation that the *E. coli* polymerase binding site in SV40 form I DNA is cleaved by S_1 nuclease (Beard *et al.*, 1973).

We have presented several lines of evidence that transcription of PM2 I involves a process of continuous chain elongation whereas transcription of PM2 II involves a process of continued initiation, polymerization, and chain termination. The evidence for these conclusions comes from the following. (a) When rifampicin is added 10 min after the start of synthesis, initiation on both templates stops immediately and synthesis on form I continues with little rate reduction, while synthesis on form II ceases within 20 min. (b) The number average chain lengths of RNA, as measured by double labeling experiments, show that the product from PM2 I grows continuously with time reaching an average size of 1.7 genome lengths (16,000 nucleotides) by 2 hr. The PM2 II product reaches a maximum size of only 0.7 genome length (7,000 nucleotides) by 30 min of synthesis and remains constant thereafter. (c) Sucrose gradient analysis also shows that the product from PM2 I grows continuously with an appreciable fraction reaching 3 genome lengths by 90 min of synthesis. The PM II product, however, reaches its maximum size within 40 min of synthesis, sedimenting in three discrete peaks at 24, 33, 39 S.

These findings indicate that the polymerase terminates transcription efficiently on form II, but very poorly on form I DNA. There are several possible mechanisms which could account for this. (1) Posttranscriptional cleavage of the PM2 II product, such as by RNase III (Dunn and Studier, 1973), seems unlikely since this is not observed with the PM2 I product. Furthermore, transcription of T7 DNA by *Pseudomonas* BAL-31 polymerase produces two major RNA products, one of 2.2×10^6 (analogous to the *E. coli* polymerase T7 transcription product; Millette *et al.*, 1970) and one of 3.1×10^6 daltons (S. G. Zimmer and R. L. Millette, manuscript in preparation). After these products are synthesized (40-min synthesis), their polyacrylamide gel profile remains unchanged after an additional 40 min of incubation in the presence of rifampicin (data not shown). (2) Termination by a ρ-like factor (Roberts, 1969) in the polymerase preparation is also considered unlikely for the above reasons. Additional evidence against this is that our *E. coli* RNA polymerase preparation, which contains no detectable ρ factor (Millette *et al.*, 1970), synthesizes RNA of the same size distribution as *Pseudomonas* BAL-31 polymerase from PM2 II DNA (Figure 6). (3) Single-strand breaks in DNA might serve as polymerase termination sites. We feel this is unlikely since our initial preparations of PM2 DNA contained an average of approximately one "nick" per molecule (from the dye-buoyant density distribution). Assuming asymmetric transcription and a random distribution of nicks, the percentage of form II molecules which contain zero, one, two, and three nicks in the coding strand is calculated to be 40, 50, 10, and 2%, respectively. This would give rise to two heterodisperse RNA distributions, one having an average size one-half the genome and the other, transcribed from the unnicked strands, having a size of greater than one genome in length. This is clearly not the distribution we find (Figure 3). Moreover, transcription of the 40% having intact coding strands should not terminate and nucleotide incorporation would not cease after rifampicin addition as we have observed. (4) Polymerase termination, such as that which takes place on T7 DNA (Millette *et al.*, 1970), seems to be the most likely explanation for termination on PM2 II. We have shown that this occurs during T7 transcription by *Pseudomonas* BAL-31 polymerase in that two discrete products of 2.2 and 3.1 megadaltons are made (S. G. Zimmer and R. L. Millette, manuscript in preparation). Furthermore, *E. coli* polymerase, which is capable of termination on T7 DNA, exhibits rifampicin sensitivity and yields the same product distribution with PM2 form II as does *Pseudomonas* BAL-31 RNA polymerase.

We have shown that RNA polymerase from *E. coli* behaves in very much the same manner as that from *Pseudomonas* BAL-31 in the transcription of the two circular forms of PM2 DNA: (a) PM2 form I DNA supports a twofold higher rate of transcription than form II; (b) transcription of only PM2 II is rifampicin sensitive (when rifampicin is added after initiation); (c) the transcription product of PM2 I is heterogeneous whereas the PM2 II product shows the same three major RNA peaks as seen with the *Pseudomonas* BAL-31 enzyme. The differences we have observed in the transcription of the two circular DNA forms of PM2 thus appear to be a function of the DNA structure and not the enzyme used. Furthermore, if it can be shown that the *E. coli* polymerase transcripts are indeed identical with those made by the BAL-31 polymerase on PM2 II, this would imply that a heterologous polymerase can recognize the same initiation and termination sites on this template.[1]

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References

Beard, P., Morrow, J. F., and Berg, P. (1973), *J. Virol. 12*, 1303.

Beerman, T. A., and Lebowitz, J. (1973), *J. Mol. Biol. 79*, 451.

Botchan, P., Wang, J. C., and Echols, H. (1973), *Proc. Nat. Acad. Sci. U.S. 70*, 3077.

Cashel, M., Lazzarini, R. A., and Kalbacher, B. (1969), *J. Chromatogr. 40*, 103.

Dausse, J., Sentenac, A., and Fromageot, P. (1972), *Eur. J. Biochem. 31*, 394.

Davis, R. W., Simon, M., and Davidson, N. (1971), *Meth-*

[1] After completing this manuscript we became aware of the results of Richardson (1974), using *E. coli* RNA polymerase, which also show that PM2-I is transcribed at a higher rate than PM2-II due to a higher rate of chain initiation, while chain growth rates on both templates are similar.

ods Enzymol. XXID, 413.
Dean, W., and Lebowitz, J. (1971), *Nature (London), New Biol. 231,* 5.
Dunn, J. J., and Studier, F. W. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 1559.
Espejo, R. T., and Canelo, E. S. (1968a), *Virology 34,* 738.
Espejo, R. T., and Canelo, E. S. (1968b), *J. Bacteriol. 95,* 1887.
Espejo, R. T., Canelo, E. S., and Sinsheimer, R. L. (1969), *Proc. Nat. Acad. Sci. U.S. 63,* 1164.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147.
Hayashi, M., Hayashi, M. N., and Spiegelman, S. (1964), *Proc. Nat. Acad. Sci. U.S. 51,* 351.
Hayashi, Y., and Hayashi, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 171.
Hayashi, Y., and Hayashi, M. (1971), *Biochemistry 10,* 4212.
Helinski, D. R., and Clewell, D. B. (1971), *Annu. Rev. Biochem. 40,* 899.
Hinkle, D. C., Ring, J., and Chamberlin, M. J. (1972), *J. Mol. Biol. 70,* 197.
Hossenlopp, P., Oudet, P., and Chambon, P. (1974), *Eur. J. Biochem. 41,* 397.
Jacob, R. J., Lebowitz, J., and Kleinschmidt, A. K. (1974), *J. Virol. 13,* 1176.
Kato, A. C., Bartok, K., Fraser, M. J., and Denhart, D. T. (1973), *Biochim. Biophys. Acta 308,* 68.
Kleinschmidt, A. K., and Zahn, R. K. (1959), *Z. Naturforsch. B 14,* 770.
Laskowski, M., Sr. (1966), *Procedures Nucleic Acid Res.,* 85.
Mandel, J.-L., and Chambon, P. (1974a), *Eur. J. Biochem. 41,* 367.
Mandel, J.-L., and Chambon, P. (1974b), *Eur. J. Biochem. 41,* 379.
Millette, R. L., and Trotter, C. D. (1970), *Proc. Nat. Acad. Sci. U.S. 66,* 701.
Millette, R. L., Trotter, C., Herrlich, P., and Schweiger, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 135.
Richardson, J. P. (1974), *Biochemistry 13,* 3164.
Roberts, J. W. (1969), *Nature (London) 224,* 1168.
Saucier, J. M., and Wang, J. C. (1972), *Nature (London) New Biol. 239,* 167.
Schweiger, M., Herrlich, P., and Millette, R. L. (1971), *J. Biol. Chem. 246,* 6707.
Sippel, A., and Hartman, G. (1968), *Biochim. Biophys. Acta 157,* 218.
Spirin, A. S. (1961), *Biokhimiya 26,* 511.
Symons, R. H. (1969), *Biochim. Biophys. Acta 190,* 548.
Takanami, M., Okamoto, T., and Sugiura, M. (1971), *J. Mol. Biol. 62,* 81.
Westphal, H. (1970), *J. Mol. Biol. 50,* 407.
Westphal, H. (1971), *in* Lepetit Colloquium on RNA Polymerase and Transcription, Silvestri, L., Ed., Amsterdam, North-Holland Publishing Co., p 77.
Zimmer, S. G., and Millette, R. L. (1975), *Biochemistry,* preceding paper.

Priming of Superhelical SV40 DNA by *Escherichia coli* RNA Polymerase for *in Vitro* DNA Synthesis†

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ABSTRACT: When closed circular SV40 DNA containing 58 negative superhelical turns is used as a template for RNA synthesis with *Escherichia coli* RNA polymerase, a fraction of the RNA product remains complexed with the DNA. The RNA in the complex is resistant to ribonuclease in high salt, and the T_m indicates that it is hydrogen bonded to the DNA. The mole ratio of RNA to DNA nucleotides in the complex ranges from 0.01 to 0.08; the RNA ranges in length from 80 to 600 nucleotides. The formation of the complex is dependent on the circular DNA being topologically underwound since no complex is formed when closed circular DNA containing zero superhelical turns is used as the template. The DNA-RNA complex can serve as a primer-template combination for *in vitro* DNA synthesis by *E. coli* DNA polymerase I. After synthesis with [α-^{32}P]-labeled deoxyribonucleoside triphosphates followed by alkaline hydrolysis, the isolation of ^{32}P-labeled ribonucleotides is evidence for a covalent linkage between the RNA and the DNA synthesized. During the *in vitro* DNA synthesis, the template is nicked at a low rate, and the nicked molecules support extensive DNA synthesis. This observation indicates that only limited synthesis can occur on unnicked molecules possibly owing to the topological constraints against unwinding of the helix. Possible models for *in vivo* priming of double-stranded DNA by *E. coli* RNA polymerase are discussed.

DNA replication is a template directed process. However, in addition to a polynucleotide template, all known DNA polymerases require a 3′-hydroxyl terminated primer chain in order to synthesize DNA *in vitro*. For this reason a closed circular double-stranded DNA cannot serve as a template for *in vitro* DNA synthesis. We sought a method to introduce a polynucleotide primer onto one or both of the strands of a closed circular DNA.

Herein we report that during RNA synthesis with *Escherichia coli* RNA polymerase, using highly superhelical

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