*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 [$\alpha$-$^{32}$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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SV40 DNA as a template, a DNA-RNA complex is formed in which the RNA is hydrogen-bonded to the DNA. We show that the RNA in the complex can provide a primer for *in vitro* DNA synthesis by *E. coli* DNA polymerase I.

## Experimental Procedures

### *Materials*

*Reagents and Chemicals.* All unlabeled ribo- and deoxyribonucleoside triphosphates, propidium diiodide, and rifampicin were obtained from Calbiochem, sodium dodecyl sulfate (SDS)[1] and density gradient grade sucrose were from Schwarz/Mann, radio tracer grade CsCl was from the Harshaw Chemical Co., formamide was from Matheson Coleman and Bell, ethidium bromide was from Boots Pure Drug Co., and all radioactively labeled compounds were from the New England Nuclear Corp.

*Media and Buffers.* Dulbecco's modified Eagle's medium was obtained from Grand Island Biological Co. Fetal calf serum was purchased from North American Biologicals.

Tris-buffered saline (TBS) is 0.137 M NaCl–5 mM KCl–0.9 mM $CaCl_2$–0.5 mM $MgCl_2$–0.7 mM $Na_2HPO_4$–25 mM Sigma 7-9 adjusted to pH 7.4. TKM is 25 mM KCl–5 mM $MgCl_2$–50 mM Tris-HCl (pH 7.5). TE buffer is 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. Standard saline citrate (SSC) is 0.15 M NaCl–15 mM sodium citrate (pH 7.0).

*Enzymes.* Pancreatic ribonuclease A was obtained from Sigma Chemical Co. and heated to 100° for 10 min in 50 mM sodium acetate buffer (pH 5.0) prior to use. Pancreatic deoxyribonuclease (electrophoretically pure) was obtained from Worthington Biochemical Corp. *E. coli* RNA polymerase, purified according to the procedure of Burgess (1969), was kindly provided by Dr. Dona Lindstrom. We are indebted to Dr. Arthur Kornberg for the preparation of purified *E. coli* DNA polymerase I.

*Virus and Cells.* The small plaque SV40 virus stock used here was obtained from Dr. Don Kiehn (Kiehn, 1973). The BSC-1 cells, passaged for some time in this laboratory, were originally obtained from E. Winocour.

*Other Materials.* Plastic petri dishes (9 cm) were purchased from A/S Nunc (Denmark). Thin-layer chromatography plates (Polygram cel 300 PEI) were obtained from Brinkman Instruments, Inc.

### *Methods*

*General.* DNA concentration was determined from the absorbance at 260 nm ($E_{260}$ 6600). Ethidium bromide concentration was determined from the absorbance at 480 nm ($E_{480}$ 5600) (Waring, 1956). Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Fractions from CsCl or sucrose gradients were dripped or aliquots spotted directly onto Schleicher and Schuell glass filters. After drying, the filters were washed extensively with 5% $Cl_3CCOOH$ containing 10 mM sodium pyrophosphate, rinsed with 95% ethanol, dried, and counted in a scintillation counter using a toluene-based scintillation fluid.

*Isolation of SV40 DNA.* BSC-1 cells, grown to confluency in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, were washed once with TBS and infected with SV40 virus at a multiplicity of <0.01 plaque forming units/cell. After allowing 60 min for adsorption, fresh medium was added. At 48 hr after infection the culture medium was replaced with medium containing either 1–2 μCi/ml of [$^3$H]thymidine (20 Ci/mmol) or 0.25 μCi/ml of [$^{14}$C]thymidine (0.05 Ci/mmol) to label the viral DNA. After 6 or 7 days of infection, prior to the release of any cells from the plate, the intracellular DNA was extracted by the method of Hirt (1967). The viral DNA was purified by phenol extraction of the Hirt supernatant followed by chloroform–isoamyl alcohol (24:1) extraction and ethanol precipitation. The ethanol precipitate was dissolved in TE, CsCl was added to make the density 1.60 g/ml, and ethidium bromide was added to give a final concentration of 300 μg/ml. The solution was centrifuged for 40 hr at 40,000 rpm and 20° in the Beckman Model 50 rotor. The DNA band was collected with the aid of a blacklight and the dye was removed by extraction with 2-propanol–$H_2O$ (9:2). The DNA was dialyzed against TE and stored at 5°. The specific activities were 1–3 × $10^4$ and 5–7 × $10^3$ cpm/μg for the $^3$H- and $^{14}$C-labeled DNAs, respectively.

*Preparation of [0] and [−58] DNAs.*[2] EXTRACT CONTAINING DNA UNTWISTING ACTIVITY. Rat liver nuclei were isolated according to the procedure of Blobel and Potter (1966). Briefly, the liver tissue was minced in TKM plus 0.25 M sucrose and then homogenized with a motor-driven Teflon pestle homogenizer. The homogenate was filtered through gauze and two volumes of 2.3 M sucrose–TKM was added. The mixture was layered over 8 ml of 2.3 M sucrose–TKM and centrifuged for 90 min in the Beckman SW27 rotor at 26,000 rpm and 5°. The nuclear pellet was washed once with TKM, resuspended in 20 mM Tris-HCl (pH 7.5)–1 mM EDTA, and sonicated with a Branson sonifier setting No. 1 until 90% of the nuclei were broken. The sonicate was centrifuged at 8000 rpm for 15 min and the supernatant stored on ice until used.

[0] DNA. The reaction mixture (1.0 ml) (Champoux and Dulbecco, 1972) contained 20 mM Tris-HCl (pH 7.5), 0.20 M KCl, 1 mM EDTA, 100 μg/ml of SV40 DNA, and 50–100 μg of extract protein. After incubation at 37° for 30 min, the reaction was stopped by making the mixture 0.2% in SDS, extracted once with phenol which had been saturated with 1 M Tris-HCl (pH 8.0), extracted once with $CHCl_3$–isoamyl alcohol (24:1), and dialyzed against TE. The DNA was purified by a CsCl–ethidium bromide equilibrium density gradient as described above for the original DNA isolation.

[−58] DNA. The procedure was identical with that for [0] DNA except that ethidium bromide was included in the reaction mixture at 8.7 μg/ml and the DNA concentration lowered to 50 μg/ml. The average number of superhelical turns was determined by the buoyant method of Gray *et al.* (1971).

*Preparation of Nicked SV40 DNA.* The reaction mixture contained 5 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 100–200 μg/ml of SV40 DNA, and 5 ng/ml of pancreatic deoxyribonuclease. After incubation

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TBS, Tris-buffered saline; TKM, 25 mM KCl–5 mM $MgCl_2$–50 mM Tris-HCl (pH 7.5); TE buffer, 10 mM Tris-HCl (pH 7.5)–1 mM EDTA.

[2] Terminology: The closed or unnicked forms of circular SV40 DNA are denoted with a prefix indicating the number of superhelical turns, *e.g.*, [−18] DNA refers to the naturally occurring form of SV40 DNA which has 18 negative superhelical turns.

for 5 min at 37° the reaction was stopped by the addition of EDTA and SDS (0.2%) and the nicked DNA purified through a CsCl-ethidium bromide gradient as described above for [0] DNA. In the preparation used in this study the initial conversion to nicked DNA was 92%. Using this value in the Poisson distribution yields a value of 2.5 for the average number of nicks per molecule.

*Conditions for RNA Synthesis.* The reaction mixture (0.10 ml) contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 0.2 mM each of ATP, CTP, and GTP and 6–33 $\mu$M unlabeled UTP plus 25 $\mu$Ci/ml of [$^3$H]UTP (27 Ci/mmol), 20 $\mu$g/ml of $^{14}$C-labeled SV40 DNA, and RNA polymerase at 30 $\mu$g/ml (ratio of enzyme to DNA approximately 10). The DNA and enzyme were preincubated for 5 min at 37° and the reaction was begun by the addition of the ribonucleoside triphosphates. The reaction was terminated by dilution with the appropriate amount of a concentrated stock of SSC buffer to give a final volume of 1.0 ml and a final concentration equal to 2 × SSC. The amount of incorporation was determined by $Cl_3CCOOH$ precipitating an aliquot on a glass filter as described above for gradient fractions. The specific activity of the synthesized RNA was calculated based on the input radioactivity and the known concentration of unlabeled UTP.

*Isolation of DNA–RNA Complex.* The products of the RNA synthesis reaction were treated with pancreatic ribonuclease at 20 $\mu$g/ml for 30 min at room temperature in 2 × SSC. SDS was added to give a final concentration of 0.5% and the mixture was extracted once with phenol, which had been equilibrated with 1 M Tris-HCl (pH 8.0), once with chloroform–isoamyl alcohol (24:1), and dialyzed against TE. CsCl was added to give a final density of 1.66 g/ml and the solution centrifuged to equilibrium in the Beckman SW56 rotor at 35,000 rpm and 20°. Gradient fractions were analyzed for radioactivity as described above.

*Thermal Dissociation of DNA–RNA Complex.* The DNA–RNA complex, in 10 mM Tris-HCl (pH 7.5)–15 mM NaCl–1 mM EDTA was sealed in glass capillary pipets which were immersed in a water bath and heated to the desired temperature. The samples were then cooled to room temperature, diluted in 2 × SSC, and treated for 30 min with 20 $\mu$g/ml of pancreatic ribonuclease. The acid-precipitable radioactivity remaining was determined as described above.

*Conditions for Formamide Dissociation of the Complex.* The samples to be dissociated were dialyzed at 5° against TE plus 90% formamide. The dialysis bag was transfered to the same buffer which had been equilibrated at 45° and left for 15 min. The formamide was removed by a final dialysis against TE.

*Neutral Sucrose Gradient Sedimentation.* The samples to be analyzed (0.20 ml) were layered over a 5–20% sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 M NaCl and centrifuged in the Beckman SW56 rotor for 2 hr at 55,000 rpm and 20 °. Gradient fractions were analyzed for radioactivity as described above.

*Conditions for DNA Synthesis.* The standard reaction mixture (Richardson *et al.*, 1964) (0.10 or 0.20 ml) contained 70 mM potassium phosphate buffer (pH 7.4), 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 mM $(NH_4)_2SO_4$, 100 $\mu$g/ml of bovine serum albumin, 30 $\mu$M each of dATP, dCTP, and dGTP, 6 $\mu$M unlabeled dTTP, and 7.5 $\mu$Ci/ml of [$^3$H]dTTP (48 Ci/mmol). The concentration of DNA and the units of enzyme added are given in the legends to the figures and tables. The amount of incorporation was determined by spotting an aliquot of the reaction mixture directly onto a glass filter which had been previously impregnated with 0.05 ml of 50 mM EDTA (to stop the reaction) and 50 mM sodium pyrophosphate. The filters were dried, washed with 5% $Cl_3CCOOH$ and ethanol, and counted.

*CsCl–Propidium Diiodide Gradient Centrifugation.* The volume of the sample to be analyzed was adjusted to 1.45 ml with TE, 1.40 g of CsCl was added, followed by 0.25 ml of a 4 mg/ml propidium diiodide stock solution (final density 1.50 g/ml). The samples were centrifuged to equilibrium in the Beckman SW56 rotor at 35,000 rpm, for 40 hr at 20°.

*Alkaline Hydrolysis and Thin-Layer Chromatography.* After synthesis with [$\alpha$-$^{32}$P]-labeled deoxyribonucleoside triphosphates, the unincorporated triphosphates were removed by exhaustive dialysis against TE containing 0.50 M NaCl followed by TE and then water. Yeast RNA was added to give a final concentration of 0.5 mg/ml and the samples were hydrolyzed in 0.30 N NaOH at 37° for 16 hr. After neutralization with HCl, the samples were applied to PEI-cellulose thin-layer plates, which had been previously washed with methanol. The two-dimensional chromatography was carried out as described by Randerath (1964) in a closed rectangular jar filled with solvent to a height of 0.8 cm. In the first dimension, the plate was developed with $H_2O$ up to the start, followed by 1.0 N formic acid up to 15 cm from the start point. The plate was dried and washed with methanol. After drying again, the plate was developed for a second time with 1.0 N formic acid and rewashed with methanol. In the second dimension the plate was developed with 60% saturated $(NH_4)_2SO_4$ up to 15 cm from the start. In this system the AMP and CMP move rapidly in the first dimension while the pyrimidine compounds move faster than the purines in the second dimension. Thus, all four ribonucleoside monophosphates are resolved and separated from any DNA which remains at the origin.

## Results

*A Complex of RNA and Superhelical SV40 DNA.* FORMATION AND ISOLATION. RNA synthesis (using $^3$H-labeled UTP) with excess *E. coli* RNA polymerase is carried out under standard reaction conditions (see Methods) utilizing $^{14}$C-labeled SV40 DNA containing either 0 or −58 superhelical turns as template. For the purification of template associated RNA we make use of the fact that RNA hydrogen bonded to DNA is resistant to pancreatic ribonuclease in high salt, whereas free single-stranded RNA is completely degraded. Following treatment of the product with ribonuclease in 2 × SSC, SDS is added and the sample is extracted with phenol and chloroform, dialyzed extensively against TE, and centrifuged to equilibrium in a CsCl density gradient. When [0] DNA is used as the template (Figure 1a), 0.26 nmol of RNA is synthesized in the reaction mixture, but there is no association of [$^3$H]RNA counts with the [$^{14}$C]DNA in the CsCl gradient. However, with [−58] DNA as the template (Figure 1b), a fraction of the 0.27 nmol of RNA synthesized is clearly associated with DNA. In this experiment 4.3% of the total RNA synthesized in the reaction is DNA associated after ribonuclease treatment. The product from the [−58] DNA template always contains ribonuclease resistant material (presumably double stranded RNA) which bands in the lower third of the CsCl gradient. In the experiments to follow, the com-

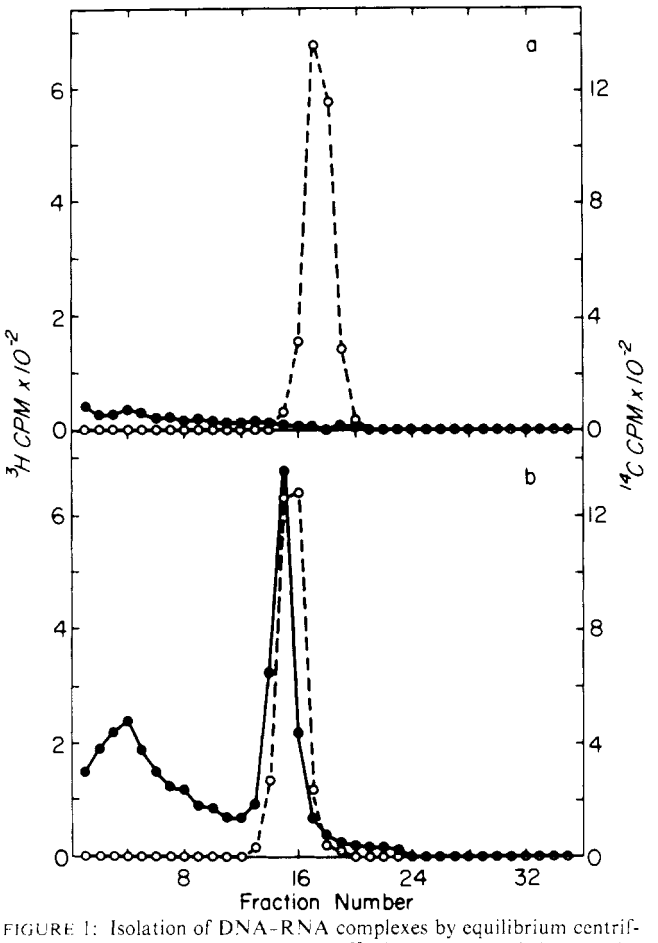


FIGURE 1: Isolation of DNA–RNA complexes by equilibrium centrifugation in CsCl. RNA synthesis with [$^3$H]UTP was carried out as described under Methods with either $^{14}$C-labeled [0] DNA (at 21 μg/ml) or [−58] DNA (at 17 μg/ml) for 5 min at 37°. There were 0.26 nmol and 0.27 nmol of RNA synthesized per 0.10 ml of reaction mixture for [0] DNA and [−58] DNA, respectively. After treatment with ribonuclease and subsequent purification (see Methods) the material from each reaction was centrifuged to equilibrium in a CsCl gradient. Density increases toward the left. The recoveries for the [0] and [−58] DNAs were 72 and 76%, respectively. (●) $^3$H-labeled RNA; (○) $^{14}$C-labeled DNA; (a) [0] DNA as template; (b) [−58] DNA as template.

plex of RNA with the superhelical DNA was always purified through a CsCl gradient as described here to eliminate this material.

It was of interest to determine whether the complex was actually formed during RNA synthesis or free RNA was hybridizing to the [−58] DNA after dissociation from the template used for its synthesis. An RNA polymerase reaction was carried out using [−18] DNA (the naturally occurring form of SV40 DNA) as a template. After 20 min of synthesis, rifampicin was added to prevent any further initiation events. At 25 min [−58] DNA was added to half of the reaction mixture and incubation was continued for an additional 20 min. The DNA-associated RNA, corrected for the ribonuclease resistant material which trails into that region of the CsCl gradient occupied by the DNA (see above), is given in Table I. It can be seen that a limited amount of complex formation occurred with [−18] DNA, the amount of DNA-associated RNA being only 6.3% that found with the same amount of [−58] DNA. However, no additional complex formed between the preexisting RNA and the [−58] DNA added after rifampicin (experiment 1). The extended incubation in the presence of rifampicin did not affect the complex already formed with the [−58] DNA (experiments 2 and 3).

CHARACTERIZATION OF THE COMPLEX. Since the specific activities of the synthesized RNA and the template DNA are known, one can determine the stoichiometry of the complex. In an experiment similar to that shown in Figure 1b, the average mole ratio of RNA to DNA nucleotides in the leading fraction of the gradient (the most dense) was 0.081 while the trailing fraction had a ratio of 0.011. The average number of RNA nucleotides per DNA molecule, calculated from these ratios, ranges from about 880 to 120. These values represent the average found for any given fraction of the gradient. It is possible that some DNA molecules on the light side of the peak contain no RNA, in which case our estimate of the amount of RNA per molecule is low.

The labeled RNA in the isolated complex was completely resistant to ribonuclease treatment in 2 × SSC, an expected result since this was one of the steps used in the purification procedure. In the absence of salt (*i.e.*, 0.01 M Tris) the RNA was quantitatively removed from the complex and rendered acid soluble by ribonuclease. The RNA thus exhibits the properties previously found for RNA hybridized to DNA (Nygaard and Hall, 1964).

In order to further characterize the association between the RNA and the DNA, the thermal dissociation profile of the complex was determined. After being heated to the desired temperature each sample was cooled to 25° and the

TABLE I: Formation of DNA–RNA Complex during Synthesis.

| Expt | Template | Treatment | Total Incub. Time (min) | [$^3$H]RNA cpm/0.1 μg of DNA |
|---|---|---|---|---|
| 1 | [−18] DNA | + Rif at 20 min | 45 | 93 |
| | | + Rif at 20 min + [−58] DNA at 25 min | 45 | 80 |
| 2 | [−58] DNA | None | 20 | 1304 |
| 3 | [−58] DNA | + Rif at 20 min | 40 | 1478 |

[a] In all three experiments the final DNA concentration during synthesis or added later was 6.7 μg/ml. Rifampicin was added to give a final concentration of 18 μg/ml. Incubation was carried out at 37° for the indicated times. The amount of [$^3$H]RNA associated with the DNA peak in the CsCl gradient was corrected for the ribonuclease resistant material which trails into the region of the CsCl gradient occupied by the DNA. The radioactivity associated with the ribonuclease resistant RNA was estimated by connecting the RNA counts on both sides of the DNA–RNA complex peak with a smooth curve (see Figure 1b).

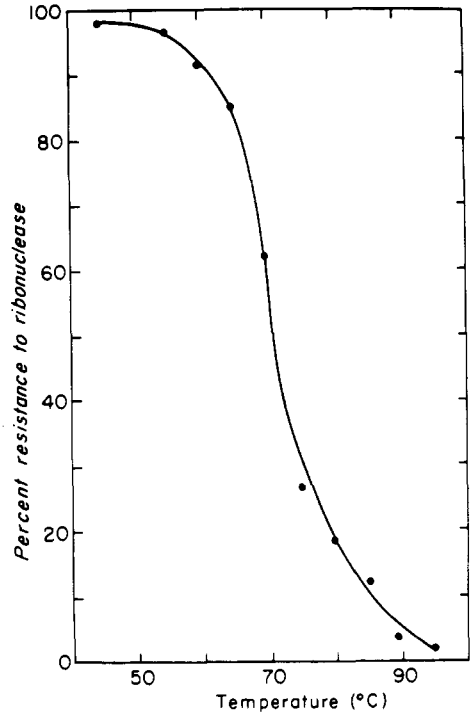


FIGURE 2: Thermal dissociation profile of the DNA-RNA complex. The DNA samples were heated in sealed capillary tubes to the indicated temperatures (see Methods). After cooling to 25°, the susceptibility of the $^3$H-labeled RNA to ribonuclease in 2 × SSC was determined. The 100% value for the [$^3$H]RNA in the unheated control corresponds to 496 cpm.

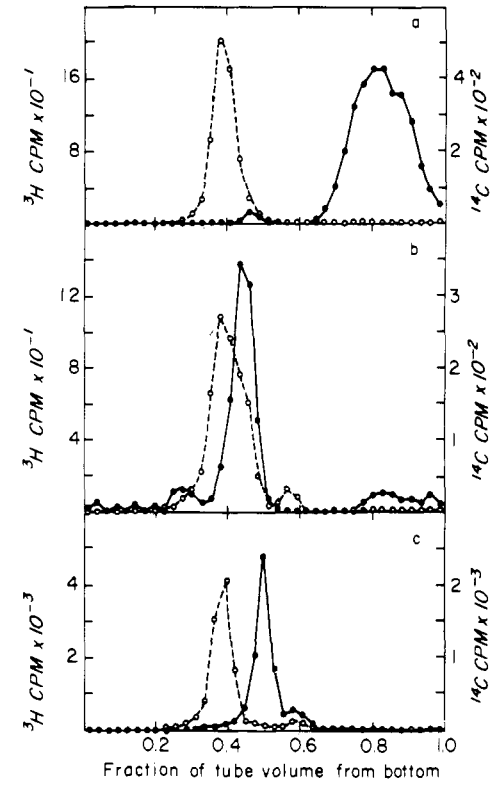


FIGURE 3: Neutral sucrose sedimentations of the intact DNA-RNA complex and of the DNA and RNA following dissociation in formamide. The isolated complex containing $^{14}$C-labeled DNA and $^3$H-labeled RNA was dissociated by heating for 15 min at 45° in 90% formamide. After removal of the formamide by dialysis the sample was layered over a 5–20% sucrose gradient and centrifuged for 2 hr at 55K rpm and 20° in the SW56 rotor. Sedimentation proceeds toward the left. (a) (●) [$^3$H]RNA; (○) [$^{14}$C]DNA, complex dissociated in formamide; (b) the undissociated complex; (c) a mixture of [−18] marker DNA (●) and [−58] DNA (○).

labeled RNA tested for its susceptibility to ribonuclease in 2 × SSC. As shown in Figure 2, a thermal dissociation profile is obtained which is characteristic of duplex nucleic acids. The midpoint of the rather broad melting transition ($T_m$) occurs at 71°.

The complex was further characterized by determining the sedimentation behavior of both the DNA and the RNA, before (Figure 3b) and after (Figure 3a) dissociation in formamide. Figure 3c shows the sedimentation profile for uncomplexed [−58] DNA compared with [−18] marker DNA (20S). Control experiments (not shown) demonstrated that the dissociation procedure (see Methods) had no effect on the sedimentation behavior of 18S and 28S rRNAs. The RNA released from the complex by formamide is heterogeneous in size (Figure 3a) with sedimentation coefficients, calculated from the positions of 20S [−18] DNA in the parallel gradient and the 4S RNA in another gradient (not shown), which range from 4 S to 10 S. These *s* values translate into an approximate length distribution of 80–600 nucleotides for the RNA in the complex (Kurland, 1960).

The unnicked [−58] DNA complexed with RNA sediments slower than uncomplexed [−58] DNA as judged by the position of the RNA counts in Figure 3b and the skewing of a fraction of the DNA into this same region of the gradient. After dissociating the RNA, the DNA returns to 25 S, the sedimentation coefficient of the uncomplexed [−58] DNA (compare Figure 3a and c).

*The RNA as a Primer for DNA Polymerase I.* KINETIC STUDIES. In order to determine whether the DNA-RNA complexes were capable of serving as primer-template combinations for DNA synthesis, we tested them in a standard synthesis with *E. coli* DNA polymerase I. The uncomplexed [−58] DNA served as a control for this experiment. We expected a limited amount of DNA synthesis in the control since all of these DNA preparations contain a small fraction (<10%) of nicked DNA, at least some of which might be used for nick translation by the polymerase (Kelly *et al.*, 1969b). As can be seen in Figure 4, the rate and extent of incorporation of [$^3$H]TTP for the DNA-RNA complexes greatly exceeded that of the control DNA (open symbols). If the complex is pretreated with ribonuclease in low salt to remove the RNA, then the synthesis is reduced to the background level of the uncomplexed DNA (closed symbols).

It is noteworthy that the kinetics of incorporation appear to be biphasic in this experiment, the initial linear rate being replaced by a slower, apparently linear rate. In other experiments, the two phases were less apparent, but in all cases the incorporation continued to increase for up to 60 min. Since we started with a template DNA which for the most part lacked single-strand nicks, we expected that after an initial burst of synthesis, further incorporation would be dependent on a nick generating system (see Discussion). Our failure to see a cessation of synthesis may mean that the template is being nicked. To test this possibility we examined the integrity of the template after a period of synthesis.

INTEGRITY OF THE TEMPLATE. Parallel reactions were set up containing either DNA-RNA complexes or uncomplexed [−58] DNA as templates and allowed to proceed for 30 min. Synthesis was terminated by the addition of EDTA and the mixtures were centrifuged to equilibrium in CsCl

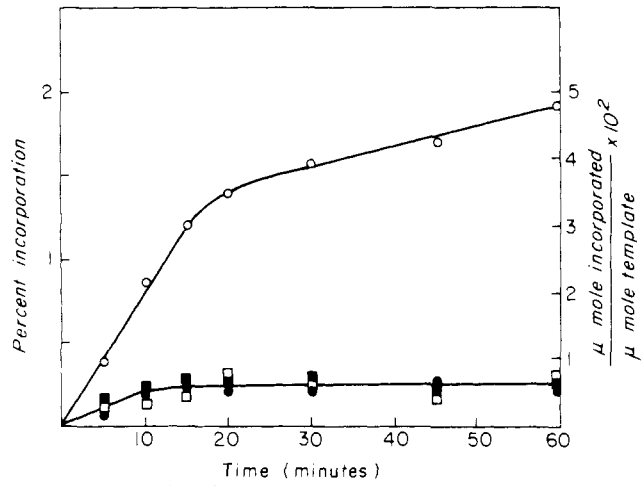


FIGURE 4: Kinetics of DNA synthesis with the DNA-RNA complex as template. DNA synthesis using DNA polymerase I was carried out as described under Methods in a total volume of 0.10 ml containing 3 μg/ml of either uncomplexed [−58] DNA (boxes) or [−58] DNA complexed with unlabeled RNA (circles) and 3.4 units of DNA polymerase. The filled symbols represent the results if the DNAs are pretreated with ribonuclease in TE prior to the DNA synthesis reaction. A blank value of 0.06% of the input radioactivity has been subtracted from each determination.

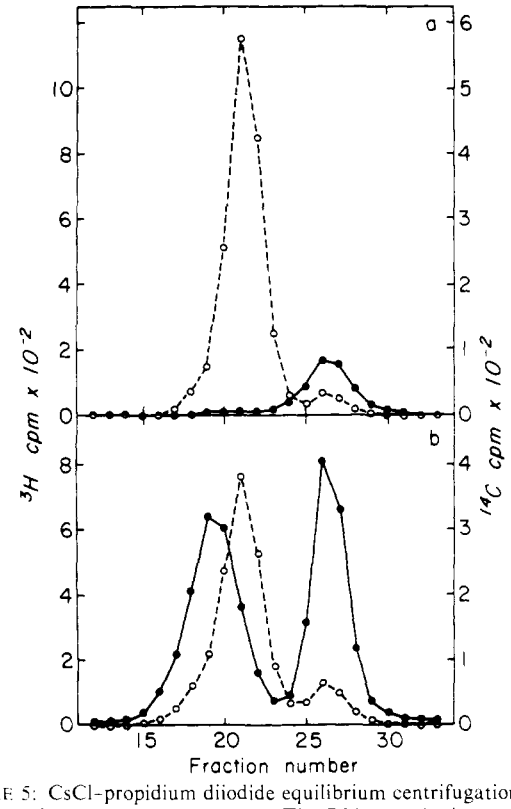


FIGURE 5: CsCl-propidium diiodide equilibrium centrifugation of the products of *in vitro* DNA synthesis. The DNA synthesis was carried out as described for Figure 4 and the products were centrifuged to equilibrium in CsCl gradients containing propidium diiodide. (●) $^{3}H$-labeled newly synthesized DNA; (○) $^{14}C$-labeled template DNA; (a) [−58] DNA as template for synthesis; (b) DNA-RNA complex as template.

gradients containing propidium diiodide to separate the closed circular DNA from any lighter nicked template. As expected, the limited amount of synthesis observed with the uncomplexed [−58] DNA was associated exclusively with the nicked component (Figure 5a) and the synthesis was complete in 10 min (see Figure 4). However, in the reaction with the DNA-RNA complexes, synthesis was observed with both the closed and the nicked forms of the DNA (Figure 5b). Moreover, the amount of newly made DNA associated with the nicked component is considerably greater than in the control reaction. An analysis of several other time points indicated that the proportion of product associated with the nicked DNA increased steadily as the length of the synthesis increased. Apparently, the template DNA is suffering at least some strand scissions during the course of synthesis in the reaction with DNA-RNA complexes. The fact that the ratio of newly made [$^{3}H$]DNA to [$^{14}C$]template is so much greater for the nicked DNA as compared to the unnicked DNA (Figure 5b) indicates that a nick increases the capacity of a molecule to act as a template for synthesis.

TABLE II: Transfer of $^{32}P$ from DNA to Ribonucleotide Monophosphates by Alkaline Hydrolysis of the Product of DNA Synthesis.

| Template for Synthesis | Cpm Transfered to | | | |
|---|---|---|---|---|
| | CMP | AMP | GMP | UMP |
| DNA-RNA complex | 119 | 51 | 81 | 93 |
| Nicked DNA | 1 | 2 | 1 | 1 |

[a] Synthesis was carried out with [$\alpha$-$^{32}P$]-labeled dCTP ($4.3 \times 10^{9}$ cpm/μmol), dATP ($8.5 \times 10^{9}$ cpm/μmol), and dTTP ($8.5 \times 10^{9}$ cpm/μmol) in a volume of 0.20 ml containing 3 μg of [−58] DNA complexed with RNA and 6.8 units of DNA polymerase. Unlabeled dGTP was added at the same concentration as the other triphosphates (0.015 mM). A parallel synthesis, carried out with nicked SV40 DNA (3 μg), was identical except the final concentration of all triphosphates was reduced to 0.0075 mM. With the DNA-RNA complex as a template a total of $1 \times 10^{-3}$ μmol of DNA was made in 45 min, while with the nicked DNA $3 \times 10^{-3}$ μmol was synthesized. After alkaline hydrolysis the same number of cpm of each sample was applied to thin-layer chromatography plates (PEI CEL 300) and developed in two dimensions to separate the ribonucleoside 2′- and 3′-monophosphates from the DNA (see Methods); $1.13 \times 10^{6}$ cpm were present at the origin in both experiments.

COVALENT LINKAGE BETWEEN SYNTHESIZED DNA AND RNA. If the RNA is indeed providing a primer terminus for DNA synthesis then alkaline hydrolysis of the product following synthesis with [$\alpha$-$^{32}P$]-labeled deoxyribonucleoside triphosphates should result in the transfer of $^{32}P$ from the 5′ side of the DNA chain to the 2′ or 3′ position of the adjacent ribonucleoside monophosphate residue. To test for such transfer we carried out a reaction in which three out of the four deoxyribonucleoside triphosphates were labeled in the $\alpha$-position with $^{32}P$. The results, given in Table II, indicate that label was transfered to all four of the ribonucleoside monophosphates when the DNA-RNA complex was used in the reaction, while no transfer occurred in a control synthesis using a template DNA which contained nicks, previously introduced by pancreatic deoxyribonuclease.

## Discussion

*The DNA-RNA Complex.* FORMATION AND STABILITY. Unlike the known DNA polymerases, RNA polymerases do initiate polynucleotide chains directly on the tem-

plate without the need for a primer. RNA synthesis probably involves the transient formation of a DNA-RNA hybrid (Hayashi, 1965). However, there is eventual dissociation of the RNA from the template with conservation of the DNA duplex. The dissociation of the product RNA from the template DNA may in fact be caused by the reformation of the DNA duplex since (1) RNA synthesis on single-stranded DNA *in vitro* yields a DNA-RNA hybrid as the product (Chamberlin and Berg, 1964; Sinsheimer and Lawrence, 1964) and (2) Chamberlin (1965) has shown that with double-stranded homopolymer templates the product of the RNA polymerase reaction is dictated by the stability of the DNA template duplex. If this duplex (DNA-DNA) is more stable than the DNA-RNA intermediate, then the RNA product is displaced, whereas if the DNA-RNA hybrid is the more stable of the two, the nontemplate DNA strand is displaced. Consistent with this model is the observation that $\phi$X DNA-DNA duplex is more stable to thermal melting than is the homologous DNA-RNA hybrid (Chamberlin and Berg, 1964). Thus, one might expect that if a circular DNA were destabilized with respect to winding of the helix, some of the product RNA would remain hydrogen bonded to the template during RNA synthesis.

The superhelical structure of isolated closed circular DNAs derives directly from the fact that the DNA is topologically underwound (Vinograd *et al.*, 1968). This topological deficiency in the number of turns means that the DNA helix is destabilized toward winding (Bauer and Vinograd, 1970a) and it was this consideration which suggested to us that the use of highly superhelical closed circular DNA (but not relaxed circles) as a template for RNA polymerase might result in some of the product RNA remaining hybridized to the DNA. The results presented here indicate that this is indeed the case.

We have shown that the formation of a complex depends on RNA synthesis using [−58] DNA as a template and does not occur as a result of the annealing of free RNA to the superhelical DNA. For this experiment we used RNA made on [−18] DNA as the source of free RNA. With this template RNA polymerase has been shown to be capable of transcribing the entire circular DNA more than once making an RNA product up to four times the length of the DNA (Delius *et al.*, 1973). Thus all the DNA sequences are represented in the RNA product, albeit asymmetrically. Therefore, the failure to find annealing of the RNA made from the [−18] DNA is not due to the absence of some DNA sequences which are present in the RNA made with [−58] DNA. We cannot rule out the unlikely possibility that some sequences of the [−58] DNA but not the [−18] DNA are transcribed with a high frequency and these later anneal with the template. It should be pointed out, however, that the conditions for synthesis (low salt, 37°) are not optimal for the reassociation of nucleic acids (Studier, 1969).

One might argue that the failure to isolate a complex of RNA with [0] DNA is not related to the formation of the complex, but rather to the unstable nature of such a complex once formed. Thus one might suppose that all DNAs initially contain a stretch of RNA hybridized to one of the DNA strands and that, during purification, the RNA is displaced essentially by branch migration (Lee *et al.*, 1970) as the DNA duplex reforms. In fact, Robberson and Clayton (1973) have shown that branch migration causes D-loop DNA displacement from nicked, but not unnicked mitochondrial DNA when the preparations are incubated under hybridization conditions. To test whether this might also happen under the conditions employed for purification of the complexes, we introduced nicks with deoxyribonuclease into a preparation of [−58] DNA containing RNA and subjected the product to our usual purification procedure. The ratio of [$^3$H]RNA to [$^{14}$C]DNA decreased from a value of 1.83 in the unnicked controls to 0.86 in the reisolated complex which had been 86% nicked. This result suggests that relief of the helix from the destabilization due to negative superhelical turns can result in some displacement of the RNA. However, a nicked complex is not directly comparable to a hypothetical complex of RNA with [0] DNA, since the latter, being constrained topologically, should have the greater tendency to cause displacement of the RNA. From these considerations it seems possible that we are measuring stability rather than formation of the complex, but further experimentation would certainly be necessary to discriminate in a definitive way between these alternatives.

NATURE OF THE COMPLEX. The buoyant density of the DNA-RNA complexes is greater than that of the DNA alone, as expected since RNA has a much greater density than DNA in CsCl (Bruner and Vinograd, 1965).

From the thermal dissociation profile of the complex we infer that the RNA is associated with the DNA by way of the usual nucleic acid base pairing through the formation of hydrogen bonds. The $T_m$ of DNA-RNA hybrids is on the order of 4-5° lower than the homologous DNA-DNA duplexes (Chamberlin and Berg, 1964). Taking this into account and the fact that we are measuring irreversible strand separation rather than continuous helix disruption (Geiduschek, 1962), we calculate from the $T_m$ of the complex that the average GC content of the DNA-RNA hybrids is about 40%. Since the GC content of SV40 DNA is approximately 41% (Crawford and Black, 1964) it appears that the hybrids have a GC content similar to that of the DNA. The breadth of the thermal transition may, however, reflect some degree of heterogeneity of the RNA with respect to its GC content.

The sedimentation coefficients of the complexes are also consistent with the proposed DNA-RNA hybrid structure. The [−58] DNA sediments faster than the viral [−18] DNA due to a more compact, supertwisted structure (Upholt *et al.*, 1971). The expected effect of having RNA hybridized to the DNA strands of [−58] DNA is for the number of superhelical turns to be lower and as a consequence the sedimentation coefficient should decrease. This was the observed result (Figure 3).

It is not possible from the data presented in Figures 1 and 3 to determine the proportion of the DNA which has RNA associated with it. A very short piece of RNA (*e.g.*, 30 nucleotides) would not be detectable due to the specific activity of the UTP used in these experiments. In addition, short pieces of RNA would be expected to have only a very small effect on the buoyant density or the sedimentation coefficient of the DNA.

The [−58] DNA helix is topologically underwound. Thus, instead of 544 helix turns[3] the molecule has only 486

[3] In calculating the number of superhelical turns ($\tau$) we have used the value of $3.6 \pm 0.3 \times 10^6$ for the molecular weight of SV40 DNA as determined by Tai *et al.* (1972). This molecular weight corresponds to 5440 base pairs. Our value for the superhelical density ($\sigma$) of SV40 DNA, determined by the buoyant method of Gray *et al.* (1971), is $-0.033 \pm 0.003$. From this value we calculate the number of superhelical turns ($\tau$) to be $-18 \pm 2$ (Vinograd *et al.*, 1968). The previously determined value of $\tau$ ($-15 \pm 2$ (Bauer and Vinograd, 1970b)) was based on a molecular weight of $3.1 \times 10^6$ and $\sigma$ equal to $-0.031 \pm 0.003$.

turns. This means that a length of DNA equal to 58 helix turns could be completely unwound while the remainder of the molecule assumes the normal secondary structure with 10 base pairs per helix turn. Fifty-eight turns corresponds to 580 nucleotides. If we assume that the RNA is hybridized to only one of the DNA strands then this represents a minimal estimate of the total possible length of RNA (as single or the sum of multiple pieces per DNA molecule) which can be found hybridized to the [−58] DNA. If RNA is associated with both DNA strands, then the total length (minimum of 2 pieces per molecule) which can be accommodated becomes 1160. These are minimal estimates since presumably more RNA could be accommodated per molecule by further unwinding of the template helix with concomitant overwinding of some other portion of the DNA (Kasamatsu *et al.*, 1971). This may not occur for the same reasons that a complex does not form with [0] DNA (see above). However, should it occur, the redistribution of helix turns, resulting in part of the DNA obtaining an excess of turns, requires an expenditure of energy which presumably could be derived from the hydrolysis of ribonucleoside triphosphates during the synthesis of the RNA (Kasamatsu *et al.*, 1971). The energy required, however, increases as the length of the RNA increases and eventually further redistribution will cease, as will chain elongation, when the energy required becomes greater than that available from polymerization.

From the mass of RNA associated with the DNA in a CsCl gradient, we have estimated the number of nucleotides of RNA per molecule of DNA to range from 120 to 880 nucleotides. The maximum does not exceed the amount that is theoretically possible. Our length estimates for the RNA, based on sucrose sedimentation, range from 80 to 600 nucleotides, the largest being near the size which we expect could easily be accommodated on the DNA strand without introducing undue strain on the molecule. These results are compatible with most molecules containing just one piece of RNA hydrogen bonded to one of the template strands. We cannot, however, estimate the frequency of molecules containing more than one RNA piece, nor can we determine whether any DNA molecules contain RNA hydrogen bonded to both strands at the same point in the DNA.

Hayashi (1965) reported the isolation of a similar DNA–RNA hybrid from RNA polymerase reactions in which $\phi$X RF DNA was used as the template. $\phi$X RF DNA has about the same number of superhelical turns as the naturally occurring form of SV40 DNA (*i.e.*, −18) (Wang, 1969). Using [−18] DNA as a template we also have detected a small amount of DNA-associated RNA (see Table I). Although a quantitative comparison is not possible, our results appear reasonably consistent with those of Hayashi (1965) and it appears likely that we are both observing the formation of DNA–RNA hybrids during RNA polymerization *in vitro*. The detection of the hybrids by Hayashi may have only been possible using a closed circular DNA containing negative superhelical turns since early attempts to detect hybrids *in vitro* with linear T2 and T4 DNA met with failure (Geiduschek *et al.*, 1961; Konrad and Stent, 1964). Our inability to detect hybrids under any conditions using [0] DNA is consistent with this interpretation.

*DNA Synthesis in Vitro.* THE RNA PRIMER. We have shown that DNA polymerase I from *E. coli* will utilize the DNA–RNA complex as a primer-template combination for *in vitro* DNA synthesis. The synthesis is dependent on the presence of the RNA since (1) the DNA–RNA complex is five times as effective as the uncomplexed DNA for synthesis and (2) removal of the RNA by ribonuclease reduces the synthesis to that of the uncomplexed DNA. Finally, we have shown that at least some of the DNA synthesized in this system is covalently linked to RNA, verifying that the RNA does indeed provide a primer terminus for polymerization. This represents the first case of *in vitro* priming of a closed circular DNA for DNA synthesis.

TEMPLATE NICKING. We have failed to detect any contaminating endonuclease activity for either double-stranded or single-stranded circular DNA in the preparation of DNA polymerase I employed in these experiments. However, during DNA synthesis with the DNA–RNA complex we do observe nicking of the DNA template. Moreover, the introduction of one or more nicks increases the net synthesis observed per molecule (see Figure 5). With uncomplexed [−58] DNA, synthesis on the small amount of nicked DNA present in the preparation ceased after 10 min. Taken together these observations mean that nuclease action (which leads to increased synthesis) either occurs as a consequence of RNA-primed synthesis or is specific for some structural feature of the DNA–RNA complex not found in the uncomplexed DNA.

DNA polymerase I is known to exhibit two kinds of exonuclease activities, one specific for the 3′-hydroxyl terminal of single- or double-stranded DNA (Lehman and Richardson, 1964) and the other which degrades double-stranded DNA specifically from the 5′ terminus (Cozzarelli *et al.*, 1969). In addition to the release of mononucleotide products, this latter activity also releases dinucleotides and other oligonucleotides at a low frequency. A special case of the 5′ activity has been described (Kelly *et al.*, 1969a) in which DNAs containing some mismatched or unpaired bases near the 5′ terminus are cleaved endonucleolytically to yield oligonucleotides. The endonucleolytic cut generally occurs at or near the point where the DNA duplex resumes. One possible interpretation of the nicking we observe during synthesis on the DNA–RNA complex is that this latter activity is introducing cuts in the nontemplate strand, which in these molecules may appear "mismatched" or be unpaired due to the strain in the molecule caused by the negative turns.

An alternative explanation for this result which we cannot at present rule out is that the DNA polymerase itself or some contaminant in the preparation specifically nicks the DNA moiety of a DNA–RNA hybrid, or is specific for some other structural feature peculiar to these molecules.

As discussed above, the closed circular DNA template should only support limited DNA synthesis due to the topological constraints against unwinding of the helix. However, any single-strand break in the unreplicated portion of the molecule should provide a swivel point for extensive synthesis. The increased synthesis observed with nicked molecules may be attributable to the nicks providing a swivel. Alternatively, the nicks may be acting as primers for nick translation (Kelly *et al.*, 1969b). At present we cannot discriminate between these two alternatives or whether both mechanisms are operative.

If synthesis on unnicked molecules is limited owing to the unwinding constraints, then synthesis should be stimulated by any nick generating system. The DNA untwisting enzyme (Champoux and Dulbecco, 1972) is an activity which has been shown to introduce a transient nick into double-stranded DNA. Since this activity might be involved in providing a swivel for DNA replication *in vivo* (Roman *et al.*, 1974) it would be of interest to test the activity for this function *in vitro*.

COMPARISON WITH *in vivo* PRIMING OF DNA SYNTHESIS. RNA synthesis has been implicated in the replication of the DNA of *E. coli* (Lark, 1972); the bacteriophages λ (Dove *et al.*, 1971), M13 (Brutlag *et al.*, 1971), and φX 174 (Schekman *et al.*, 1972); the plasmids colEl (Clewell and Evenchik, 1973) and Fl (Kline, 1973); the mitochondria from HeLa and mouse cells (Grossman *et al.*, 1973); and polyoma virus (Hunter and Francke, 1974; Magnusson *et al.*, 1973). RNA has been shown to be directly involved in the priming of DNA chains in *E. coli* (Sugino *et al.*, 1972; Sugino and Okazaki, 1973) and in the *in vitro* replication of M13 DNA (Wickner *et al.*, 1972), φX 174 DNA (Schekman *et al.*, 1972), and polyoma DNA (Hunter and Francke, 1974; Magnusson *et al.*, 1973).

Of particular interest here is the finding that the conversion of M13 single-stranded DNA to double-stranded RF (replicative form) and the subsequent replication of the RF *in vivo* is inhibited by rifampicin (Brutlag *et al.*, 1971). Therefore the *E. coli* RNA polymerase (or at least the β subunit which is the rifampicin sensitive component (Zillig *et al.*, 1970)) is involved not only in the synthesis of the phage RF from single-strand DNA, but also in the replication of the double-strand RF molecules. With single-stranded DNA as a template, the RNA product remains hydrogen bonded to DNA and in effect primes the template for DNA synthesis (assuming only part of the DNA is hybridized to RNA). However, since the double-stranded RF replication also requires RNA polymerase, it appears that at least under some circumstances, the enzyme can carry out synthesis on double-stranded DNA in which the RNA product remains template bound.

We have shown that if the DNA helix is destabilized with respect to winding, a fraction of the RNA product is not displaced from the template. A similar reaction could account for priming by *E. coli* RNA polymerase *in vivo*. The driving force for unwinding could be derived from the DNA being topologically underwound as in our *in vitro* system, or by proteins which bind tightly to single-stranded DNA. The DNA unwinding proteins, for which the T4 gene 32 protein is the prototype (Alberts and Frey, 1970), are good candidates for this role.

References


Alberts, B. M., and Frey, L. M. (1970), *Nature (London) 227,* 1313–1318.

Bauer, W., and Vinograd, J. (1970a), *J. Mol. Biol. 47,* 419–435.

Bauer, W., and Vinograd, J. (1970b), *J. Mol. Biol. 54,* 281–298.

Blobel, G., and Potter, U. (1966), *Science 154,* 1662–1665.

Bruner, R., and Vinograd, J. (1965), *Biochim. Biophys. Acta 108,* 18.

Brutlag, D., Schekman, R., and Kornberg, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2826–2829.

Burgess, R. R. (1969), *J. Biol. Chem. 244,* 6160–6167.

Chamberlin, M. J. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24,* 1446–1457.

Chamberlin, M., and Berg, P. (1964), *J. Mol. Biol. 8,* 297–313.

Champoux, J. J., and Dulbecco, R. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 143–146.

Clewell, D. B., and Evenchik, B. G. (1973), *J. Mol. Biol. 75,* 503–513.

Cozzarelli, N. R., Kelly, R. B., and Kornberg, A. (1969), *J. Mol. Biol. 45,* 513–531.

Crawford, L. V., and Black, P. H. (1964), *Virology 24,* 388–392.

Delius, H., Westphal, H., and Axelrod, N. (1973), *J. Mol. Biol. 74,* 677–687.

Dove, W. F., Inokuchi, H., and Stevens, W. F. (1971), *in* The Bacteriophage Lambda, Hershey, A. D., Ed., New York, N.Y., Cold Spring Harbor Laboratory, pp 747–771.

Geiduschek, E. P. (1962), *J. Mol. Biol. 4,* 467–487.

Geiduschek, E. P., Nakamoto, T., and Weiss, S. B. (1961), *Proc. Nat. Acad. Sci. U. S. 47,* 1405–1415.

Gray, H. B., Jr., Upholt, W. B., and Vinograd, J. (1971), *J. Mol. Biol. 62,* 1–19.

Grossman, L. I., Watson, R., and Vinograd, J. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3339–3343.

Hayashi, M. (1965), *Proc. Nat. Acad. Sci. U. S. 54,* 1736–1743.

Hirt, B. (1967), *J. Mol. Biol. 26,* 365–369.

Hunter, T., and Francke, B. (1974), *J. Mol. Biol. 83,* 123–130.

Kasamatsu, H., Robberson, D. L., and Vinograd, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2252–2257.

Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A. (1969a), *Nature (London) 224,* 495–501.

Kelly, R. B., Cozzarelli, N. R., Deutscher, M. P., Lehman, I. R., and Kornberg, A. (1969b), *J. Biol. Chem. 245,* 39–45.

Kiehn, E. D. (1973), *Virology 56,* 313–333.

Kline, B. (1973), *Biochem. Biophys. Res. Commun. 50,* 280–288.

Konrad, M. W., and Stent, G. S. (1964), *Proc. Nat. Acad. Sci. U. S. 51,* 647–653.

Kurland, C. G. (1960), *J. Mol. Biol. 2,* 83–91.

Lark, K. G. (1972), *J. Mol. Biol. 64,* 47–60.

Lee, C. S., Davis, R. W., and Davidson, N. (1970), *J. Mol. Biol. 48,* 1–22.

Lehman, I. R., and Richardson, C. C. (1964), *J. Biol. Chem. 239,* 233–241.

Lowry, O. H., Rosebrough, A. L., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Magnusson, G., Pigiet, V., Winnacker, E. L., Abrams, R., and Reichard, P. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 412–415.

Nygaard, A. P., and Hall, B. D. (1964), *J. Mol. Biol. 9,* 125–142.

Randerath, K. (1964), *Experientia 20,* 406–407.

Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., and Kornberg, A. (1964), *J. Biol. Chem. 239,* 222–232.

Robberson, D. L., and Clayton, D. A. (1973), *J. Biol. Chem. 248,* 4512–4514.

Roman, A., Champoux, J. J., and Dulbecco, R. (1974), *Virology 57,* 147–160.

Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. L., and Kornberg, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2691–2695.

Sinsheimer, R. L., and Lawrence, M. A. (1964), *J. Mol. Biol. 8,* 289–296.

Studier, F. W. (1969), *J.Mol. Biol. 41,* 199–209.

Sugino, A., Hirose, S., and Okazaki, R. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1863–1867.

Sugino, A., and Okazaki, R. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 88–92.

Tai, H. T., Smith, C. A., Sharp, P. A., and Vinograd, J. (1972), *J. Virol. 9,* 317–325.

Upholt, W. B., Gray, H. B., Jr., and Vinograd, J. (1971), *J.*

*Mol. Biol. 62,* 21-38.
Vinograd, J., Lebowitz, J., and Watson, R. (1968), *J. Mol. Biol. 33,* 173-197.
Wang, J. C. (1969), *J. Mol. Biol. 43,* 263-272.
Waring, M. J. (1956), *J. Mol. Biol. 13,* 269-282.
Wickner, W., Brutlag, D., Schekman, R., and Kornberg, A. (1972), *Proc. Nat. Acad. Sci. U.S. 69,* 965-969.
Zillig, W., Zechel, K. Rabussay, D., Schachner, M., Sethi, V. S., Palm, P., Heil, A., and Seifert, W. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 47-58.

# Effects of 5-Azacytidine on Nucleolar RNA and the Preribosomal Particles in Novikoff Hepatoma Cells†

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ABSTRACT: Examination of nucleolar RNA from cultured Novikoff hepatoma cells treated for 3 hr with $5 \times 10^{-4}$ M 5-azacytidine shows that significant amounts of analog-substituted 45S RNA are processed to the 32S RNA species, but 28S RNA formation is completely inhibited. Under these conditions of analog treatment 37% of the cytidine residues in the 45S RNA is replaced by 5-azacytidine. During coelectrophoresis of nucleolar RNA from 5-azacytidine-treated and control cells, the analog-substituted 45S RNA and 32S RNA display reduced mobilities compared to the control 45S RNA and 32S RNA. Coelectrophoresis of analog-substituted and control RNA after formaldehyde denaturation shows no differences in electrophoretic mobility between the two RNA samples, suggesting that 5-azacytidine incorporation may alter the secondary structure of the 45S RNA and the 32S RNA. 5-Azacytidine at $5 \times 10^{-4}$ M severely inhibits protein synthesis in Novikoff cells by 3 hr. After this length of treatment, however, CsCl buoyant density analysis reveals no difference in density of either the 80S or 55S preribosomal ribonucleoprotein particles when compared to normal particles. Also 5-azacytidine treatment does not appear to cause major changes in the polyacrylamide gel electrophoresis patterns of the proteins in the 80S and 55S preribosomal particles. These results together with previous findings suggest that 5-azacytidine's inhibition of rRNA processing is possibly related to its alteration of the structure of the ribosomal precursor RNAs and is not a consequence of a general inhibition of ribosomal protein formation.

In mammalian cells rRNA is formed by a unique maturation process that occurs within the nucleolus. The mature 28S and 18S RNAs are formed from a large initial precursor, the 45S RNA. The 45S RNA becomes methylated and undergoes a sequence of specific cleavages forming various intermediate RNA species and finally the mature rRNAs (Weinberg *et al.,* 1967; Weinberg and Penman, 1970; Attardi and Amaldi, 1970; Burdon, 1971). rRNA processing does not occur with free RNA molecules. Studies have indicated that the ribosomal precursor RNAs exist as ribonucleoprotein complexes (Liau and Perry, 1969; Warner and Soeiro, 1967). Proteins become attached to the 45S RNA during or immediately after its synthesis. Warner and Soeiro (1967) were able to isolate discreet nucleolar RNP[1] particles designated as 80 S and 55 S based on their sedimentation properties and found these particles to contain 45S and 32S RNA, respectively.

At present little is known about the cleavage enzymes involved in processing of ribosomal precursor RNA or how the cell regulates the maturation process. Several factors are known to be necessary for normal processing. Proper methylation is important. During maturation the methylated portions of the 45S RNA are conserved (Wagner *et al.,* 1967). Processing is inhibited if the ribosomal precursor RNAs are undermethylated (Vaughan *et al.,* 1967). In addition to methylation, studies have shown a close correlation between rRNA processing and protein synthesis. Cycloheximide decreases 45S RNA synthesis and inhibits its processing in HeLa cells (Willems *et al.,* 1969). Puromycin also alters rRNA maturation (Soeiro *et al.,* 1968). HeLa cells starved for the essential amino acid, L-valine, display reduced rRNA processing (Maden *et al.,* 1969). When HeLa cells were incubated in hypertonic media which causes a gradual inhibition of protein synthesis the first effect on ribosome synthesis observed was reduced processing of the 45S RNA (Pederson and Kumar, 1971).

In our laboratory we have been interested in the effects of base analogs on the processing of rRNA. Numerous analogs of both purine and pyrimidine bases have been found to inhibit rRNA maturation (Perry, 1965; Tavitian *et al.,* 1968; Wilkinson *et al.,* 1971; Wilkinson and Pitot, 1972; Weiss and Pitot, 1974a,b). Evidence suggests that the analogs must be incorporated into the ribosomal precursor RNAs in order to exert their inhibitory effect (Tavitian *et*

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[1] Abbreviations used are: RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate; 5-azaCR, 5-azacytidine; RSB buffer, 0.01 M NaCl-0.0015 M $MgCl_2$-0.01 M Tris-HCl (pH 7.4); NEB, 0.01 M NaCl-0.01 M EDTA-0.01 M Tris-HCl (pH 7.4).