RNA-Dependent DNA Polymerase Activity of RNA Tumor Viruses

II. Directing Influence of RNA in the Reaction

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The role of ribonucleic acid (RNA) in deoxyribonucleic acid (DNA) synthesis with the purified DNA polymerase from the avian myeloblastosis virus has been studied. The polymerase catalyzes the synthesis of DNA in the presence of four deoxynucleoside triphosphates, Mg^{2+} , and a variety of RNA templates including those isolated from avian myeloblastosis, Rous sarcoma, and Rauscher leukemia viruses; phages f2, MS2, and Q β ; and synthetic homopolymers such as polyadenylate-polyuridylic acid. The enzyme does not initiate the synthesis of new chains but incorporates deoxynucleotides at 3' hydroxyl ends of primer strands. The product is an RNA-DNA hybrid in which the two polynucleotide components are covalently linked. Free DNA has not been detected among the products formed with the purified enzyme *in vitro*. The DNA synthesized with avian myeloblastosis virus RNA after alkaline hydrolysis has a sedimentation coefficient of 6 to 7S.

Ribonucleic acid (RNA) tumor viruses contain an enzyme which catalyzes the synthesis of deoxyribonucleic acid (DNA) from RNA (3, 26). The products include RNA-DNA hybrids, single-stranded DNA, and double-stranded DNA. The DNA product is of low molecular weight and is composed of sequences complementary to the viral RNA (8, 9, 22, 24). In addition to RNA-primed activity, the polymerase also catalyzes a DNA-dependent DNA synthesis (9, 25).

In the preceding paper (14), the mechanism of DNA priming of deoxynucleotide incorporation was examined. These studies indicated that both avian myeloblastosis virus (AMV) and Rauscher leukemia virus (RLV) polymerases catalyzed repair-like reactions and that the polymerases were unable to initiate *de novo* new DNA chains. These polymerases resembled DNA polymerase II isolated from *Escherichia coli* in their priming requirements as well as their products.

In the present communication, the viral polymerases have been studied by using RNA primers. Evidence will be presented that the viral polymerases are unable to initiate DNA chains with RNA primers. The products formed in RNA-primed systems are RNA-DNA hybrids in which polydeoxynucleotide chains are covalently attached to the RNA.

MATERIALS AND METHODS

AMV was a generous gift of J. Beard, Duke University, and RLV was obtained from The Pfizer Co., Inc. Maywood, N.J.

Q β RNA was a gift from G. Kuo of this institution; MS2 RNA was from Miles Laboratories, Inc.; purified *E. coli* tRNA^{Met} was a gift from L. Schulman; f2 RNA was a gift from U. Maitra; and Rous sarcoma virus (RSV) RNA was a gift from P. Duesberg, University of California, Berkeley.

Mung bean nuclease I (15, 17) was generously provided by M. Laskowski, Sr. This enzyme was dissolved in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5) before use.

All other materials were obtained as described previously (14).

Assay of RNA-primed reactions. Unless otherwise indicated, reaction mixtures (0.05 ml) contained: 1 μ mole of Tris-hydrochloride (pH 8.0), 0.5 μ mole of $MgCl_2$, 0.25 μ mole of KCl, 0.3 μ mole of dithioerythritol, 5 nmoles each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP), 1 nmole of 3H -deoxythymidine triphosphate (dTTP; 50 to 8,000 counts per min per pmole), and RNA and polymerase as indicated. The reaction was stopped after 30 min of incubation at 38 C by the addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.02 ml of denatured salmon sperm DNA (2.6 μ moles/ml), and 5% trichloroacetic acid. After 5 min at 4 C, the precipitate was collected on Gelman type E glass fiber filters, dried, and counted in 10 ml of toluene scintillation fluid in a

scintillation counter. The following procedure was used to reduce the blank values when products were collected from mixtures with deoxynucleoside triphosphates of high specific activity (8,000 to 10,000 counts per min per pmole). Reaction mixtures as described above were treated with 0.1 ml of 0.1 M sodium pyrophosphate, 0.05 ml of denatured salmon sperm DNA (2.6 μ moles/ml), 0.8 mg of serum albumin, and 2 ml of 5% trichloroacetic acid. After 5 min at 4 C the precipitate was pelleted by centrifugation at $10,000 \times g$ for 2 min in an International refrigerated centrifuge, and the supernatant was discarded. The precipitate was dissolved in 0.5 ml of 1 M NH_4OH , reprecipitated with 5% trichloroacetic acid, collected, and counted on glass fiber filters as described above.

Assay of ribonuclease activity. Ribonuclease was assayed in a volume of 0.05 ml containing: 2.5 μ moles of Tris-hydrochloride (pH 8.0), 0.5 μ mole of MgCl_2 , 0.25 μ mole of dithioerythreitol, 0.25 μ mole of KCl, 0.12 A_{260} ^3H -poly uridylic acid (U) (7×10^4 counts/min), and enzyme. After incubation at 38 C for 30 min, 0.125 μ mole of denatured salmon sperm DNA, 0.8 mg of albumin, and 0.5 ml of 5% trichloroacetic acid were added; the tubes were placed in ice for 5 min and then centrifuged for 2 min at $10,000 \times g$ in an International refrigerated centrifuge. The supernatant fluid containing acid-soluble materials was counted in 10 ml of Bray's (4) scintillation fluid.

Gradient centrifugation assay for ribonuclease activity. To assay for ribonuclease, 1.4 nmoles of ^3H -f2 RNA (6,000 counts/min) was incubated under the conditions for the DNA polymerase assay with the labeled deoxynucleoside triphosphate omitted. After incubation, the reaction mixture was brought to a final concentration of 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.4% sodium dodecyl sulfate (SDS), and 3% formaldehyde in a volume of 0.25 ml; heated to 65 C for 15 min; and layered on a 5-ml 5 to 20% linear sucrose gradient containing 0.2% SDS, 3% formaldehyde, 20 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 0.1 M NaCl. The gradient was centrifuged at $300,000 \times g$ for 240 min at 20 C in a Spinco SW65 rotor. Thirty-drop fractions were collected directly into counting vials containing 10 ml of Bray's scintillation fluid. When ^3H -RLV RNA (0.15 nmole; 2,000 counts/min) was used instead of ^3H -f2 RNA, the procedure was the same except that gradients were centrifuged at $180,000 \times g$ for 2 hr in a Spinco SW65 rotor.

Preparation of AMV RNA. AMV (250 mg) was pelleted in a Spinco 40 rotor at $105,000 \times g$ for 30 min. The virus was suspended in 10 ml of 0.1 M NaCl, 40 mM sodium phosphate (pH 7.0), and 0.1 mM EDTA (buffer A) and extracted four times by the phenol-SDS method described by Duesberg (5) except that bulk *E. coli* tRNA (1 optical density unit per ml) was added as a carrier. The RNA was precipitated by the addition of two volumes of 95% ethanol, suspended in 1 ml of buffer A, and precipitated with two volumes of 95% ethanol. The precipitate was dried in vacuo and dissolved in 0.2 ml of buffer A, and the RNA was sedimented at $180,000 \times g$ in a Spinco SW65 rotor for 60 min at 4 C on a 5 to 20% sucrose

gradient in buffer A. The RNA in the 60S region of the gradient was pooled and then precipitated with two volumes of 95% ethanol. The precipitate was collected by centrifugation at $105,000 \times g$ in a Spinco SW65 rotor for 60 min, and the RNA was dissolved in 20 mM NaCl, 0.1 mM EDTA, 20 mM sodium phosphate buffer (pH 7.0) and stored frozen at -10 C in small portions.

Preparation of ^3H -RLV RNA. Tritium-labeled RNA was prepared from RLV isolated from incubation of chronically infected mouse CL1 cells adapted to suspension culture (using an unpublished method developed by R. Bases of this institution) with ^3H -uridine (2 Ci/mmol; 2.5 nmoles/ml) for 18 hr. Virus was purified by isopycnic banding in sucrose, and RNA was extracted and prepared as above. These preparations were carried out in collaboration with R. Bases.

Equilibrium density gradient centrifugation in Cs_2SO_4 solution of DNA polymerase product treated with or without formaldehyde. A ^3H -labeled product containing 7.1×10^4 counts/min in 0.5 ml was treated with 0.5 ml of phenol neutralized to pH 8.0; the aqueous phase was collected and again shaken with phenol; the combined phase was dialyzed for 3 hr against $0.1 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl} + 0.015 \text{ M sodium citrate}$). Samples (0.1 ml) were added to 2 ml of 0.02 M sodium phosphate buffer (pH 7.0) containing or lacking 0.25 M HCHO. The solutions containing formaldehyde were heated at 85 C for 10 min and then cooled quickly. Solid Cs_2SO_4 (2.6 g) was added; the solution was diluted to 4 ml with $4 \times \text{SSC}$ and centrifuged in polyallomer tubes for 44 hr at 38,000 rev/min in an SW39 rotor. Fractions were collected from a hole pierced in the bottom of the tube, refractive indexes were measured, and the amount of acid-insoluble radioactivity was determined. ^3H -poly (U) and ^3H -d(AT) copolymer markers were run in different tubes.

Equilibrium density gradient centrifugation of poly (A)·poly (U) polymerase product. Reaction mixture (0.1 ml) contained 3.7 nmoles of $\alpha^{32}\text{P}$ -dTTP (8,400 counts per min per pmole), 10 μg of albumin, 5 μ moles of Tris-hydrochloride buffer (pH 8.0), 0.2 μ mole of MnCl_2 , 20 nmoles of poly (A), 0.6 nmole of poly (U) (average chain length 70), and 0.08 unit of RLV polymerase (phosphocellulose eluate). After 30 min at 38 C, reactions were stopped by the addition of 0.9 ml of SSC and 1 ml of neutralized aqueous phenol. The aqueous phase was dialyzed for 12 hr against 250 ml of SSC yielding 2.6×10^6 counts/min of acid-insoluble ^{32}P . A portion was heated with HCHO as follows: 0.05 ml of ^{32}P product was added to a solution (2.95 ml) containing 10 μ moles of sodium phosphate buffer (pH 7.0), 500 μ moles of HCHO, and 1 nmole of ^3H -poly (U). The mixture was heated at 75 C for 10 min and then quickly cooled in an ice-water bath. To the formaldehyde-treated preparation, 0.6 ml of $10 \times \text{SSC}$ and 2.6 g of solid Cs_2SO_4 were added, and the mixture was centrifuged in polyallomer tubes at 36,000 rev/min in an SW39 rotor for 60 hr at 20 C. Fractions were collected after a hole was pierced in the bottom of the tube, refractive indexes were measured, and the amount of acid-insoluble

radioactivity was determined. The recovery of ^{32}P in the fractions counted was 72%.

Poly (A)·poly (U) transfer experiments. Reaction mixture (0.1 ml) contained 0.8 nmole of $\alpha^{32}\text{P}$ -dTTP (1,700 counts per min per pmole), 10 μg of albumin, 10 μmoles of Tris-hydrochloride (pH 8.0), 0.1 μmole of MnCl_2 , 28 nmole of poly (A) and 2.9 nmole of poly (U) (average chain length 50 nucleotides), 5 μmoles of 2-mercaptoethanol, and 1.67 Kornberg units of DNA polymerase I or 2.1 units of Rauscher polymerase, or 0.38 unit of AMV polymerase. Reactions were incubated for 60 min at 30 C, carrier DNA (0.02 ml of salmon sperm; 2.6 $\mu\text{moles}/\text{ml}$) was added, and the precipitate was collected by centrifugation. The pellet was dissolved in 0.2 ml of 0.3 N NH_4OH , acidified with trichloroacetic acid, and centrifuged. This procedure was repeated two more times, and the final pellet was dissolved in 0.5 N KOH. The amount of acid-insoluble radioactivity observed was 9.54×10^5 , 2.54×10^6 , and 3.5×10^4 counts/min for reaction mixtures containing DNA polymerase I, RLV polymerase, and AMV polymerase, respectively. The solution was incubated for 22 hr at 37 C, after which time 0.02 ml of salmon sperm carrier DNA was added. This was followed by acidification with HCl. The pellet was collected by centrifugation, and the supernatant fluid was adsorbed to Norite (0.1 ml of 10%). The Norite was washed twice with water, and the adsorbed material was eluted three times with 0.5-ml portions of 50% ethanol in 1.5 N NH_4OH . The ethanolic- NH_3 eluates were concentrated to dryness and dissolved in water. The amount of ^{32}P -labeled nucleotide recovered at this point was: 10,720, 1,560, and 720 counts/min from reactions containing DNA polymerase I, RLV polymerase, and AMV polymerase, respectively. Samples (4,960, 1,250, and 520 counts/min from the DNA polymerase I, RLV, and AMV polymerase mixtures, respectively) with carrier 2'(3') mononucleotides were subjected to electrophoresis on Whatman 3 MM paper saturated with 0.05 M sodium citrate, pH 3.5, for 1.5 hr at 6,000 v. The areas corresponding to the ultraviolet-absorbing regions were cut out, eluted, and counted in Bray's scintillation fluid. No ^{32}P was detected in regions of the electropherogram between the ultraviolet-absorbing regions, nor was there any ^{32}P located at the origin. A small amount of ^{32}P possibly representing di- or trinucleotides, etc. was detected ahead of the 2'(3') uridine monophosphate (UMP) region.

The radioactivity eluted from the 2'(3') UMP region was subjected to the action of alkaline phosphatase or crude snake venom (*Crotalus adamanteus*). In the former case, all of the ^{32}P was rendered Norite nonadsorbable, whereas in the latter case 90% of the ^{32}P remained Norite adsorbable. These observations indicate that the ^{32}P in the 2'(3') UMP region is not present in TpT or oligonucleotides.

Viral polymerase preparations. The DNA polymerase activities of AMV and RLV were purified extensively from crude detergent extracts, utilizing poly d(AT) copolymer as primer (14). Enzyme preparations of specific activity greater than 3 $\mu\text{moles}/\text{mg}$ of protein were employed. In most of the experiments described below, the AMV polymerase was used. With this enzyme preparation, deoxyribonuclease and

approximately 90 to 95% of ribonuclease activities present in crude extracts were removed after passage through the phosphocellulose column (pH 8.6). The remaining ribonuclease activity, measured with ^3H -poly (U), was separated from the polymerase by sedimentation in glycerol gradients. The AMV polymerase was estimated to have a molecular weight of approximately 160,000 and sedimented more rapidly than ribonuclease activity. The enzyme activity obtained after glycerol gradient centrifugation will be referred to as purified polymerase pI. Although purified polymerase pI was found to be free of ribonuclease when this activity was measured employing ^3H -poly(U), ribonuclease activity was detected when the polymerase preparations (0.4 unit) were incubated with ^3H -RLV or ^3H -f2 RNA preparations and the RNA species were subjected to formaldehyde denaturation. Under these conditions, a decrease in the sedimentation value of the labeled RNA was detected in sucrose gradients. Presumably, these RNA preparations contain enough secondary structure so that nicks introduced by ribonuclease do not change their sedimentation properties unless their secondary structure is destroyed.

AMV polymerase preparations containing no detectable ribonuclease activity were prepared by chromatography of the phosphocellulose pH 8.6 eluate on a second phosphocellulose column at pH 7.0. The elution of the enzyme activity was carried out as previously described (14) with the exception that 0.04 M sodium phosphate buffer (pH 7.0) was used in place of Tris buffer. Such polymerase preparations obtained in 60% yield will be referred to as purified polymerase pII. Incubation of 0.25 unit of AMV polymerase pII with ^3H -f2 RNA did not alter the sedimentation profile in formaldehyde-sucrose gradients.

RESULTS

Influence of enzyme concentration on rate of DNA synthesis. The influence of enzyme concentration on RNA-primed DNA synthesis was studied by using AMV RNA as primer (Fig. 1). As shown, a sigmoidal curve was obtained which reached a plateau at 20 ng of protein. This sigmoidal relationship was also observed with DNA primers. These results suggest that the AMV polymerase may be composed of subunits which tend to dissociate at low protein concentration (18), though other interpretations are possible. The amount of enzyme used in these experiments was capable of incorporating 62 pmoles of deoxynucleotides with poly d(AT) as primer under the standard assay conditions described in the preceding paper (14). The low activity observed with AMV RNA as primer is due to its limiting concentration in the reaction. Reactions containing 0, 200, 400, and 1,000 pmoles of AMV RNA resulted in the incorporation of <0.2, 5.9, 9.4, and 31.4 pmoles of deoxynucleotides, respectively. In all of the experiments presented

FIG. 1. AMV RNA-dependent DNA polymerase activity. AMV RNA (206 pmoles) was incubated with ^3H -dTTP (8,000 counts per min per pmoles) under the conditions described in the text with varying concentrations of purified AMV polymerase pI as indicated.

below, limiting amounts of viral RNA were used because of our limited supply.

Duesberg et al. (7) have shown that during purification of the polymerase from RSV there is no separation of DNA- and RNA-primed activities. We have obtained similar results with AMV and RLV polymerases. Heat inactivation studies with the AMV polymerase indicate that polymerase activity with either DNA or RNA primers is inactivated at the same rate. For example, when AMV polymerase preparations were incubated for 3 min at 28, 44, 60, and 65 C, nucleotide incorporation measured with AMV RNA (206 pmoles) as primer was found to be 5.6, 4.3, 2.0, and <0.2 pmoles in 30 min, respectively; deoxynucleotide incorporation with d(AT) copolymer (4.9 nmoles) as primer was found to be 104, 80, 32, and 0.9, respectively, for these preparations. In each case, the rate of inactivation of polymerase activity with AMV RNA or d(AT) copolymer as template was the same. These results support the idea that one enzyme is responsible for both activities.

Requirements of RNA-primed reactions. The requirements for the synthesis of DNA with AMV RNA as template are summarized in Table 1. The purified polymerase pII required magnesium ions for activity; optimal activity occurred at 10 mM Mg^{2+} , and the requirement for Mg^{2+} could be partially met by Mn^{2+} (1 mM). In polymerase preparations contaminated with ribonuclease, such as polymerase pI, the activity with Mn^{2+} was greater than with Mg^{2+} alone; 0.2 mM Mn^{2+} gave optimum activity. However, with either enzyme preparation when magnesium and manganese ions were both present, there was a greater rate of deoxynucleotide incorporation

TABLE 1. Requirements for synthesis of DNA with avian myeloblastosis virus (AMV) polymerase^a

Additions	Deoxynucleotide incorporation (pmoles/30 min)
Complete.....	1.13
Omit MgCl_2	<0.02
Complete + MnCl_2 (1 mM).....	1.65
Complete - MgCl_2 + MnCl_2 (1 mM).....	0.63
Complete - KCl.....	0.96
Complete - dithioerythritol.....	0.20
Complete - RNA.....	<0.02
Complete + ribonuclease (2 μg).....	<0.02
Complete + deoxyribonuclease (10 μg).....	0.05

^a Conditions of the polymerase assay were as described in the text with 67 pmoles of AMV RNA, 0.2 unit of purified polymerase pII, ^3H -deoxycytidine triphosphate (4,460 counts per min per pmoles) and unlabeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate.

than was observed with either cation alone. The polymerase required the presence of sulfhydryl reagents such as dithioerythritol or mercaptoethanol for activity and possessed a broad pH optimum between pH 7.8 and 9.0, with maximum activity observed at pH 8.2; deoxynucleotide incorporation at pH values of 9.8 and 7.2 was 50% of that observed at pH 8.2. When RNA was omitted from the incubation mixture, no detectable deoxynucleotide incorporation occurred. Similarly, the addition of either pancreatic ribonuclease or deoxyribonuclease inhibited DNA synthesis (Table 1). In another series of experiments, it was shown that AMV RNA-primed synthesis depended on the presence of all four deoxynucleoside triphosphates for maximal activity (Table 2); the omission of a single deoxynucleotide markedly reduced DNA synthesis. Furthermore, all four deoxynucleotides were incorporated in approximately equimolar amounts (Table 3).

Specificity of DNA polymerase for RNA templates. A number of RNA preparations supported deoxynucleotide synthesis with purified polymerase preparations in agreement with results obtained by Duesberg et al. (*unpublished data*). Viral RNA isolated from RSV and RLV stimulated deoxythymidine monophosphate (dTMP) incorporation to about the same extent as AMV RNA (Table 4).

Several RNA preparations from sources other than RNA tumor viruses were tested. Of these, f2, MS2, Q β , and bulk *E. coli* transfer RNA (tRNA) preparations were active templates for

TABLE 2. *Effect of omission of deoxynucleoside triphosphates on DNA synthesis^a*

Additions	Deoxynucleotide incorporation (pmoles/30 min)
Complete	1.60
Omit dATP, dGTP, dCTP	0.18
Omit dATP	0.24
Omit dGTP	0.39
Omit dCTP	0.28

^a Conditions for assay were as described in the text except that 0.2 nmole each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP), 0.03 nmole of ³H-dTTP (8,200 counts per min per pmole), 206 pmoles of avian myeloblastosis virus RNA, and 0.35 unit of purified polymerase pI were added.

TABLE 3. *Incorporation of labeled deoxynucleotides by avian myeloblastosis virus (AMV) polymerase^a*

Labeled deoxynucleotide added	Deoxynucleotide incorporation (pmoles/30 min)
³ H-dTTP	0.31
³ H-dGTP	0.28
³ H-dCTP	0.39
³ H-dATP	0.29

^a Four separate reaction mixtures were prepared with 67 pmoles of AMV RNA and 0.2 unit of purified polymerase pII in which each one contained one of the deoxynucleoside triphosphates labeled with tritium. In these reactions 1 nmole of ³H-deoxynucleoside triphosphate was present with 5 nmoles each of the unlabeled deoxynucleoside triphosphates. The specific activities of the labeled deoxynucleoside triphosphates were: ³H-deoxythymidine triphosphate (dTTP; 8,210 counts per min per pmole), ³H-deoxyguanosine triphosphate (dGTP; 1,600 counts per min per pmole), ³H-deoxycytidine triphosphate (dCTP; 4,460 counts per min per pmole), and ³H-deoxyadenosine triphosphate (dATP; 7,090 counts per min per pmole).

the synthesis of DNA in the presence of Mn²⁺ and Mg²⁺, whereas a purified preparation of *E. coli* tRNA_f^{Met} was not. The efficiency of priming with these RNA preparations, however, was markedly lower than that observed with tumor viral RNA species.

The native conformation of the tumor viral RNA preparations is disrupted by heating at 80°C in low ionic strength buffers (2, 6, 10). This treatment results in an irreversible change in the sedimentation coefficient of the RNA species from 70 to 30S and, as found by Duesberg et al. (*unpublished data*), a marked decrease in tem-

TABLE 4. *Comparison of template activities of different RNA preparations^a*

Expt	RNA added ^b	Amt of RNA added (pmoles)	Deoxynucleotide incorporation (pmoles/30 min)	
			Mg ²⁺	Mg ²⁺ + Mn ²⁺
I	AMV	206	3.6	9.6
	RSV	500	5.2	11.2
	RLV	150	1.1	6.9
	f2	10,000	0.9	43.5
	Q β	1,650	0.2	8.5
	Bulk <i>Escherichia coli</i> tRNA	8,700	1.5	5.6
	<i>E. coli</i> tRNA _f ^{Met}	7,800		0.14
II	AMV	206	2.6	8.3
	MS2	242	0.08	0.77
	Q β	237	0.28	1.05
	f2	150	0.22	1.73
	bulk <i>E. coli</i> tRNA	260	0.45	0.33
	<i>E. coli</i> tRNA _f ^{Met}	390		0.08

^a Purified avian myeloblastosis virus (AMV) polymerase pI (0.35 unit) was incubated with different RNA preparations and ³H-deoxythymidine triphosphate (8,200 counts per min per pmole) in the standard DNA polymerase assay. Mn²⁺ (0.2 mM) was added where indicated. In the experiments reported above, all DNA synthesis obtained with any of the RNA primers was quantitatively inhibited by the addition of ribonuclease (2 μ g).

^b Abbreviations: RSV, Rous sarcoma virus; RLV, Rauscher leukemia virus; tRNA, transfer ribonucleic acid.

plate activity. RSV and AMV RNA species were tested after heat treatment and found to be active (Table 5). Ten to 30% of the template activity was retained when assayed in the presence of Mg²⁺, whereas 50 to 75% of the template activity was observed when assayed in the presence of Mn²⁺ and Mg²⁺.

Extent of DNA synthesis. The extent of DNA synthesis with AMV RNA as primer was investigated (Fig. 2). As shown, the yield of deoxynucleotide incorporation was equivalent to 3% of the added RNA primer. The observed plateau was not due to inactivation of enzyme activity during the incubation since the addition of more enzyme at 20 min did not further increase deoxynucleotide incorporation. The addition of more RNA, on the other hand, resulted in an immediate resumption of deoxynucleotide incorporation which again reached the plateau corresponding to 3% of the added RNA. These results show that only a limited amount of deoxynucleotide can be incorporated with a given amount of RNA. In

TABLE 5. Effect of heat denaturation on priming activity of avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) RNA preparations^a

RNA added	Amt of RNA added (pmoles)	Deoxynucleotide incorporation (pmoles/30 min)	
		Mg ²⁺	Mg ²⁺ + Mn ²⁺
AMV.....	206	1.13	1.59
AMV heated.....	206	0.40	0.80
RSV.....	400	1.32	1.45
RSV heated.....	400	0.15	1.10

^a Where indicated, RNA preparations were heated at 80 C for 3 min and rapidly cooled; additions were as described in Table 1 with purified polymerase pII (0.2 unit).

FIG. 2. Kinetics of deoxynucleotide incorporation with AMV RNA and DNA polymerase. Assay conditions were as described in the text with ³H-dCTP (4,460 counts per min per pmoles), 67 pmoles of AMV RNA, and 0.375 unit of purified polymerase II added. After 20 min of incubation, an additional 67 pmoles of AMV RNA or 0.15 unit polymerase pII was added. Symbols: (●) deoxynucleotide incorporation; (○) deoxynucleotide incorporation after addition of RNA at 20 min; (▲) deoxynucleotide incorporation after addition of enzyme at 20 min.

other experiments (not shown) conducted under conditions described in Fig. 2, the addition of ribonuclease (2 μg) at 20 min had no effect on the yield of DNA synthesis.

Susceptibility of Rauscher RNA to nuclease and its effect on template activity. The RNA from RNA tumor viruses sediments in neutral sucrose gradients with a sedimentation coefficient of 60 to 70S (8, 20, 21). Denaturation by treatment with dimethyl sulfoxide, urea, or heat (2, 6, 10) reduces the sedimentation coefficient to 20 to 30S and smaller materials, suggesting that the native RNA is composed of pieces hydrogen-bonded together. Further support for such a structure

has been presented by Bader and Steck (2) who observed that the 60 to 70S Rauscher RNA remains intact after action of endogenous viral ribonuclease; upon denaturation, small RNA species (<20 to 30S) were detected.

We investigated the structure of the Rauscher RNA by studying its susceptibility to attack by several nucleases. Tritium-labeled RLV RNA was rendered acid-soluble after incubation with pancreatic ribonuclease (Table 6). This result suggests that the secondary structure of the RNA does not involve long stretches of complementary sequences such as those found with the duplex reovirus RNA. In addition, complex structures such as poly (A)·poly (U) are also susceptible to pancreatic ribonuclease attack under the conditions employed (Table 6).

The *Neurospora* and mung bean I nucleases (15, 17, 19) specifically degrade single-stranded but not duplex structures of DNA. These enzymes also attack single-stranded regions of RNA in RNA duplexes or single-stranded regions of RNA and DNA in RNA-DNA hybrids. As shown in Table 6, ³H-poly (U) is completely susceptible to the action of *Neurospora* and mung bean nucleases, after annealing to poly (A), ³H-poly (U) was completely resistant to the nucleases. In other experiments, RNA-DNA hybrids were formed with fd DNA and RNA polymerase and isolated by gradient density banding in Cs₂SO₄. The RNA in such structures is resistant to *Neurospora* nuclease; heat denaturation of the hybrids renders the RNA susceptible to this nuclease preparation.

When ³H-RLV RNA was treated with either mung bean or *Neurospora* nucleases, approximately 50% of the RNA remained acid-insoluble. These results suggest that there is considerable internal structure of the RNA, in accord with the previously reported observations (2, 6, 8, 10, 20, 21, Duesberg et al., unpublished data). Upon heat denaturation, 90% of the RNA was susceptible to *Neurospora* nuclease action, suggesting a marked disruption of the internal structure of the RNA. The interpretation of the latter finding is complicated by the presence of nicks in the ³H-RLV RNA. Approximately 50 to 60% of the ³H-RLV RNA in our preparations sedimented slower than 20S after heat denaturation. Nicks present in the RNA would yield structures incapable of reversible denaturation if, for example, hair-pin structures existed in intact RNA. A diagrammatic representation is shown in Fig. 3.

The single-stranded regions of the AMV RNA were shown to be essential for template activity by use of the *Neurospora* nuclease. The nuclease was preincubated with AMV RNA for 10 min at 38 C to degrade single-stranded regions. After

heating at 65 C for 4 min to inactivate the nuclease activity, deoxynucleoside triphosphates and polymerase were added and the rate of DNA synthesis was measured (Table 7). AMV RNA

TABLE 6. Susceptibility of Rauscher leukemia virus (RLV) RNA and poly (U) to enzymatic digestion^a

RNA	Treatment	Acid-insoluble RNA (%)
³ H-RLV RNA	Ribonuclease (4 µg)	<5
	<i>Neurospora</i> nuclease (0.02 unit)	56
	<i>Neurospora</i> nuclease (0.04 unit)	43
	Mung bean nuclease I (0.2 unit)	51
	Pancreatic deoxyribonuclease (10 µg)	93
	RNA heated at 100 °C for 3 min; <i>Neurospora</i> nuclease (0.02 unit) added	13
³ H-poly (U)	Ribonuclease (4 µg)	<2
	<i>Neurospora</i> nuclease (0.04 unit)	<2
	Mung bean nuclease I (0.2 unit)	<2
	Poly (A) + ribonuclease (4 µg)	7
	Poly (A) + <i>Neurospora</i> nuclease (0.04 unit)	>98
	Poly (A) + mung bean nuclease I (0.2 unit)	>98

^a Tritium-labeled RLV RNA (80 pmoles, 2,340 counts per min) was incubated in reaction mixtures (0.1 ml) containing 5 µmoles of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 0.5 µmoles of MgCl₂, and nucleases as indicated. ³H-poly (U) (7 nmoles, 62,000 counts per min) and poly (A) (10 nmoles) were added where indicated, and reaction conditions were the same as those described with RLV RNA. In reaction mixtures treated with mung bean nuclease I, the polynucleotides as indicated were added to solutions containing MgCl₂. After mixing, potassium acetate buffer (pH 5.1; 2 µmoles) and enzyme were added. All other conditions were as described above.

FIG. 3. Diagrammatic representation of hairpin structures in RNA. The region designated with a bar represents complementary sequences.

TABLE 7. Effect of *Neurospora* nuclease treatment of avian myeloblastosis virus (AMV) RNA^a

Treatment of AMV RNA	Deoxynucleotide incorporation (pmoles)
None.....	1.13
Heated at 65 C.....	0.80
<i>Neurospora</i> nuclease (0.02 unit)....	<0.10

^a AMV RNA (206 pmoles) was incubated with *Neurospora* nuclease at 38 C for 10 min. The reaction mixtures were heated to 65 C for 4 min to inactivate the nuclease and then incubated under the DNA polymerase assay conditions described in Table 1.

which was preincubated without the nuclease retained 70% of its activity compared to non-preincubated controls. On the other hand, RNA preincubated with the nuclease did not prime DNA synthesis. Similar structural requirements for single-stranded regions have been demonstrated for DNA-primed DNA synthesis as previously reported (14).

Characteristics of DNA polymerase product. The nature of product of the AMV DNA polymerase and AMV RNA was examined by treatment with various chemical and enzymatic reagents. As reported by others, the labeled product possessed characteristics expected of DNA. It was resistant to hydrolysis by heating at 100 C at pH 6.5 or in 1 M KOH but was completely converted to an acid-soluble form after heating in 1 N HCl. The product was resistant to digestion by ribonuclease and quantitatively attacked by deoxyribonuclease (Table 8). After treatment with alkali, the labeled product banded at a density of 1.45 in Cs₂SO₄ solution characteristic of DNA. The alkali-treated product had a sedimentation coefficient of 6 to 7S measured in alkaline sucrose gradients with single-stranded ¹⁴C-labeled fd DNA as a reference marker. This represents a molecular weight of approximately 1.7×10^5 to 2.6×10^5 . The size of the DNA was the same whether it was synthesized in the presence of Mg²⁺ and Mn²⁺ or Mg²⁺ alone.

Product as an RNA-DNA hybrid. Duesberg et al. (7) and Faras et al. (12) have reported that newly synthesized DNA formed with purified polymerase and RSV RNA cosediments with 60 to 70S viral RNA in neutral sucrose gradients. We repeated this experiment with ³H-RLV or AMV RNA preparations with similar results. A small portion of the ³²P-labeled DNA was found at the top of the gradient presumably due to the presence of ribonuclease in the polymerase preparation used. These results suggest that the immediate polymerase product is an RNA-DNA

TABLE 8. Enzymatic digestion studies on DNA polymerase product^a

Treatment of product	Amt of product remaining acid-insoluble (%) in expt		
	I	II	III
Ribonuclease (4–20 μ g).....	100	100	
Deoxyribonuclease (4 μ g).....	<5	<5	
<i>Neurospora</i> nuclease 0.01 unit.....			74
0.02 unit....	67	78	
0.04 unit....	54	52	57
Mung bean nuclease I 0.2 unit....			88
1.0 unit....			70
Heat at 100 C for 5 min followed by:			
<i>Neurospora</i> nuclease (0.04 unit).....	22		21
Mung bean nuclease I (0.2 unit).....			44
Mung bean nuclease I (1.0 unit).....			33
Heat at 100 C for 7 min in 1 M KOH followed by:			
<i>Neurospora</i> nuclease (0.04 unit).....	5		10
Mung bean nuclease I (1 unit).....			8

^a Products were prepared with avian myeloblastosis virus RNA (206 pmoles) and polymerase pI as described in Table 4. Products were dialyzed to remove deoxynucleotides and then incubated in 0.1 ml containing 5 μ moles of Tris (hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 0.5 μ mole of $MgCl_2$, and enzymes as indicated. After 30 min at 38 C, the amount of acid-insoluble radioactive product was measured. In each of these experiments, approximately 1,500 counts per min corresponding to 5.7 pmoles of DNA product were used. In reaction mixtures containing alkaline hydrolyzed product, the KOH was removed with Dowex 50 (H^+) before addition of the *Neurospora* nuclease or mung bean nuclease I. In all experiments carried out with the latter enzyme, the conditions described in Table 6 were used.

hybrid. This has been shown to be correct by the following observations: (i) the majority of the ³H-dTMP-labeled product was found to be resistant (55 to 80%) to digestion by *Neurospora* or mung bean I nucleases (Table 8). After the product was treated with alkali to digest RNA, between 5 and 10% of the DNA was resistant to *Neurospora* or mung bean I nucleases; (ii) the dTMP-labeled product banded at a density of 1.835 in $CsCl$ solution which is characteristic of RNA (Fig. 4), whereas less than 2% banded as free DNA. These experiments indicate that the product, under the conditions employed, is mostly, if not exclusively, an RNA·DNA hybrid.

FIG. 4. Equilibrium $CsCl$ solution gradient centrifugation of DNA polymerase product. A DNA product formed with AMV RNA as described in Fig. 5 was used for $CsCl$ solution gradient centrifugation. The sample was diluted to 2.1 ml with $2\times$ SSC, and 2.8 g of solid $CsCl$ was added, yielding a density of 1.799. Centrifugation and conditions were as described in Fig. 5. Symbols: (○) tritium-labeled product; (●) density.

Covalent attachment of the RNA to DNA. It was previously reported (14; Leis and Hurwitz, Fed. Proc. 30:1153, 1971) that the Rauscher and AMV DNA polymerases were incapable of initiating synthesis of new chains with poly d(AT) copolymer as template. Deoxynucleotide incorporation occurred at the 3'-OH end of a primer strand, and all newly synthesized product was covalently attached to the poly d(AT) copolymer primer. We therefore investigated the nature of the linkage between the AMV RNA and the DNA in the hybrid product. If RNA is covalently attached to the DNA, then the two components should be inseparable by density gradient centrifugation after denaturation in the presence of formaldehyde. A ³H-dTMP-labeled product was prepared (see above) and divided into two equal portions. One half was heated at 85 C for 10 min in 0.25 M formaldehyde and the other sample was unheated. Both products were then analyzed by Cs_2SO_4 solution equilibrium density gradient centrifugation with ³H-poly(U) and ¹⁴C-T7 DNA as density markers. The untreated product banded as a broad peak at densities intermediate to that of free DNA and RNA (Fig. 5A). This heterogeneity would be expected for RNA·DNA hybrids possessing varying amounts of the two components. The product heat-denatured in formaldehyde also banded at a hybrid density (Fig. 5B); no detectable product banded at the density of free DNA, indicating that under the conditions employed all of the DNA product was covalently linked to RNA. (Similar denaturation experiments have been carried out with

FIG. 5. Equilibrium Cs_2SO_4 solution gradient centrifugation of DNA polymerase product. (A) A polymerase product (2.1×10^4 counts/min) was prepared with AMV RNA (206 pmoles) and 0.2 unit purified polymerase pI, dialyzed against SSC, and extracted with phenol. A portion was sedimented to equilibrium in Cs_2SO_4 solution for 44 hr at $123,000 \times g$ as described in the text. The recovery of ^{32}P was 71%. (B) A portion of the polymerase product was heated in 0.25 M formaldehyde at 85 C for 10 min and sedimented to equilibrium in formaldehyde Cs_2SO_4 solution as above. The recovery of ^{32}P was 66%. Symbols: (○) ^{32}P -dTMP-labeled product; (●) density.

RNA-DNA hybrids formed with *E. coli* DNA-dependent RNA polymerase. ^{32}P -products formed with ^3H -labeled single-stranded DNA were isolated by CsCl gradient centrifugation and then subjected to HCHO denaturation as described above. Under these conditions, quantitative separation of RNA and DNA was achieved. In these experiments, the average chain of RNA was approximately 400 nucleotides.) The shift in density observed in formaldehyde-heated preparations is most likely due to the single-strand breaks present in the 60 to 70S AMV RNA primer which results in products containing a higher proportion of DNA.

The covalent linkage of the DNA product to the RNA is not limited to the viral RNA template. Similar observations were made with the

synthetic polynucleotides poly (A)·poly (U), as well as with phage RNA f2. ^{32}P -dTMP-labeled polymerase products were prepared with ^3H -f2 RNA in the presence of Mn^{2+} (see above). One-half of the product was heated at 65 C for 15 min in 3% formaldehyde, and both products were sedimented through a neutral SDS-sucrose gradient. Formaldehyde was included in the gradient containing the heated product. The ^{32}P -labeled product cosedimented with the ^3H -labeled f2 RNA before and after the formaldehyde treatment (Fig. 6), indicating that the DNA was covalently linked to the f2 RNA. Similar experiments with ^3H -RLV RNA as template were performed. There was general correspondence between the sedimentation profile of ^{32}P -DNA and ^3H -RNA. A sizeable amount of the RNA, however, did not cosediment with the ^{32}P -DNA after formaldehyde denaturation, most likely due to the presence of nicks in the 60 to 70S RNA. In the absence of formaldehyde treatment, ^3H - and ^{32}P -labeled materials cosedimented as previously discussed.

The synthetic homopolymers, poly (A)·poly (U), in the presence of Mn^{2+} specifically support the synthesis of poly(dT) as reported by Scolnick et al. (23). The product poly(dT) formed in such reaction mixtures banded in Cs_2SO_4 solution as a sharp peak at the density of 1.52 (Fig. 7B). ^3H -poly(U) and ^3H -poly(dT) added to separate gradients as standard markers banded at a density of 1.66 (Fig. 7) and 1.43, respectively. After heat denaturation in formaldehyde, the ^{32}P -product still banded at 1.520 (see above) indicating covalent attachment of the poly(dT) to the RNA primer. The covalent attachment of ^{32}P -poly(dT) to poly (U) was further demonstrated by subjecting ^{32}P -poly(dT) products to alkaline hydrolysis. If ^{32}P -dTMP is covalently attached to poly (U), alkaline hydrolysis should result in the transfer of ^{32}P to UMP residues yielding ^{32}P -labeled 2'(3')UMP. A poly(dT) product was prepared with several enzymes; DNA polymerase I from *E. coli*, AMV, and RLV DNA polymerases (see above). After alkaline hydrolysis, carrier 2'(3')-mononucleotides were added and separated by high-voltage electrophoresis. The area of the electrophoregrams corresponding to 2'(3') UMP contained 86, 90, and 92%, respectively, of ^{32}P label in the mononucleotide regions (Table 9). Further substantiation that the ^{32}P -product present in these regions contained 2'(3') phosphomonoester structure was obtained; all of the ^{32}P was converted to a Norite-nonadsorbable form with alkaline phosphatase. In contrast, 5' nucleotidase (crude snake venom) did not affect the Norite-adsorbable property of the ^{32}P -product. The radioactivity

FIG. 6. Gradient centrifugation of DNA polymerase product formed with f2 RNA as primer. (A) A DNA polymerase product was prepared in 0.1 ml with 76 nmoles of ^3H -labeled f2 RNA (338 counts per min per nmole), $\alpha^{32}\text{P}$ -dTTP (5,000 counts per min per pmole), 0.5 mM MnCl_2 , and 0.28 unit of purified polymerase pII as described in the text. After 15 min of incubation at 38 C, the reaction mixture was brought to a final concentration of 0.1 M EDTA and 0.4% SDS in a volume of 0.25 ml; layered onto a 5-ml 5 to 20% linear sucrose gradient in 0.2% SDS, 20 mM sodium phosphate (pH 7), 1 mM EDTA, and 0.1 M NaCl; and centrifuged at $180,000 \times g$ for 80 min at 20 C in a Spinco SW65 rotor. Thirty-drop fractions were collected by gravity after piercing the bottom of the centrifuge tube, precipitated with 5% trichloroacetic acid, collected on glass fiber filters, and counted in a scintillation counter. Untreated tritium-labeled f2 RNA (30S) sedimented in a separate gradient at the same rate as the treated f2 RNA. (B) A DNA polymerase product prepared as above was heated to 65 C for 15 min in 3% formaldehyde after addition of EDTA and SDS. The product was analyzed as above except that the sucrose gradient contained 3% formaldehyde and was centrifuged at $300,000 \times g$ for 240 min. The formaldehyde-treated f2 RNA sedimented with a sedimentation coefficient of 13S. Symbols: (○) ^3H -f2 RNA; (●), ^{32}P -dTMP-labeled product. The ratio of ^{32}P to ^3H for each experiment is summarized in the inserts in the above figures.

FIG. 7. Equilibrium Cs_2SO_4 solution gradient centrifugation of DNA polymerase product prepared with poly (A)·poly (U). DNA polymerase product labeled with ^{32}P -dTTP, heated at 75 C for 10 min with 0.25 M formaldehyde, and sedimented to equilibrium in Cs_2SO_4 solution as described in text. Symbols: (○), $\alpha^{32}\text{P}$ -dTTP-labeled product; (●), density.

detected in the Ap region was not further characterized and may represent aberrant TMP incorporation to the poly (A) chain.

DISCUSSION

In the presence of four deoxynucleoside triphosphates and Mg^{2+} , the purified viral enzymes catalyze repair-like reactions on RNA, DNA, or RNA-DNA hybrids. Polymerase ac-

tivities primed by these different templates were always found in the same fractions during enzyme purification and were inactivated by heat at the same rate, suggesting that one enzyme is capable of using these different templates for DNA synthesis. A similar conclusion has been reached by Duesberg et al. (7). The incorporation of deoxynucleotides occurred at approximately the same rate with an equal amount of either DNA or RNA as template with the AMV polymerase. Furthermore, AMV polymerase primed deoxynucleotide incorporation with DNA or RNA templates was identically sensitive to a variety of inhibitors. [Thymus DNA (nicked and treated with exonuclease III)-stimulated deoxynucleotide incorporation was inhibited 51% by anthramycin (0.13 mM), 98% by aurin tricarboxylic acid (0.34 mM), 90% by lucanthone (0.19 mM), and 99% by Congo red (0.12 mM), whereas camptothecin (0.1 mM) had no effect. AMV RNA-stimulated deoxynucleotide incorporation was inhibited 46, 96, 73, 97, and 0%, respectively, by the same inhibitors.]

The mechanism of deoxynucleotide incorporation with RNA primers appears to be the same as that found with DNA primers (14). With DNA primers, as well as synthetic RNA-DNA hybrids, evidence had been presented that

TABLE 9. Distribution of ^{32}P in mononucleotides after alkaline hydrolysis^a

2'(3') Mononucleotide	DNA polymerase system used		
	DNA polymerase I	AMV	Rauscher virus
Uridylate	86	90	92
Guanylate	<2	<2	<3
Adenylate	12	8	4
Cytidylate	<2	<2	<2

^a Polymerase product was prepared in reactions primed with poly (A)·poly (U) as described in the text labeled with 5' α - ^{32}P -deoxythymidine triphosphate. The product was hydrolyzed with alkali, and the mononucleotides were separated by high-voltage electrophoresis in 0.05 M sodium citrate (pH 3.5). The amount of ^{32}P counted corresponded to 4,400 counts/min (DNA polymerase I), 450 counts/min [avian myeloblastosis virus (AMV) polymerase] and 1,120 counts/min (Rauscher virus polymerase). Control experiments were carried out with ^{32}P -labeled poly (dT) synthesized in reaction mixtures primed with poly (A)·poly (dT). The product (1.09×10^5 counts/min) carried through the above procedure did not lead to the isolation of detectable nucleotides (<80 counts/min).

deoxynucleotide incorporation occurs at the 3'-OH end of primer strands attached to template strands. These structures must possess single-stranded regions to direct synthesis as represented in Fig. 8. Thus, when single-stranded regions of AMV RNA were degraded with *Neurospora* nuclease, this RNA no longer supported deoxynucleotide incorporation. As expected, the DNA synthesized with AMV RNA was largely resistant to the action of *Neurospora* and mung bean nucleases, indicating its duplex structure. In reactions primed with poly (A)·poly (U) (in the presence of Mn^{2+}), the rate of dTMP incorporation was markedly increased by the use of poly(U) previously nicked with micrococcal nuclease followed by alkaline phosphatase treatment. Decreasing the poly(U) average chain length from 200 to 300 nucleotides to 50 increased the rate of dTMP incorporation 10- to 20-fold. Furthermore, the alkaline transfer experiments (Table 9) indicate that the 3'-OH end of the poly(U) chain is the site of dTMP incorporation.

The DNA formed in the presence of AMV RNA is an RNA·DNA hybrid whose polynucleotide content is mostly RNA. It bands in CsCl and Cs_2SO_4 solutions at densities expected of products rich in RNA, and no labeled product was detected banding at a density of free DNA.

FIG. 8. Deoxynucleotide incorporation occurring at 3'-OH end of primer strands attached to template strands. Arrow denotes direction of deoxynucleotide incorporation.

As observed in reactions primed with DNA polymers, the polymerase does not initiate synthesis of new chains de novo with RNA primers but incorporates deoxynucleotides onto the 3'-OH ends of the primer strands. Thus, the newly synthesized DNA is covalently linked to the RNA primer in a hybrid structure. This attachment was demonstrated by the banding of AMV RNA-polymerase products in Cs_2SO_4 solution in the hybrid density regions after treatment with formaldehyde. Similarly ^{32}P -dTMP-labeled products cosedimented with ^3H -f2 RNA primer-template in neutral sucrose-SDS gradients both before and after treatment with formaldehyde.

Only about 3% of the AMV RNA added as primer is transcribed into DNA. The size of the in vitro synthesized DNA was determined to be 6 to 7S by sedimentation in alkaline sucrose gradients, equivalent to a molecular weight of approximately 1.7×10^5 to 2.6×10^5 . DNA isolated after incubation of partially permeable virus with labeled deoxynucleoside triphosphates was found to have similar sedimentation coefficients (8, 9). At present, it is not known whether the DNA synthesized in our system represents a heterogeneous DNA population complementary to the entire RNA genome as reported by Duesberg et al. (9) for virion-generated DNA.

Since viral RNA primers support a limited extent of DNA synthesis, it appears that the DNA polymerase is unable to denature the secondary structure of RNA. This limited activity is similar to the activity found with the T4-induced DNA polymerase and DNA polymerase II of *E. coli*. With these two enzymes, specific proteins have been shown to play an important role in repair reactions. The T4 gene 32 protein specifically stimulates the T4-induced DNA polymerase (1; B. Alberts et al., Fed. Proc. 30:1036, 1971), and a protein recently isolated from *E. coli* specifically stimulates DNA polymerase II in repair of DNA (M. Gefter and T. Kornberg, Fed. Proc. 30:1110, 1971). Similar proteins may be present either in virions or in infected cells and could facilitate viral polymerase transcription of RNA.

Manly et al. (16) and Fanshier et al. (11) reported that both single- and double-stranded DNA free of RNA are produced by detergent-treated virions. As of yet, we have not detected free DNA products in our purified system *in vitro* but rather only RNA·DNA hybrids. Thus we believe that factors present in the virion participate in the formation of free DNA. Possible mechanisms yielding free and double-stranded DNA from RNA·DNA hybrids might include the following: (i) the covalent linkage between DNA and RNA could be hydrolyzed by an endonuclease in the virion. This cleavage would be followed by denaturation of the DNA chain from the RNA by a concerted process involving transcription and peeling as postulated by Manly et al. (16). Since the viral polymerase does not act at single-strand breaks (at least with DNA), this model requires localized denaturation which could be catalyzed by a T4-gene 32-like protein. It is equally possible that both mechanisms occur concomitantly. (ii) Inherent in the model of the primer-template role that viral RNA plays is the importance of hair-pin structures. If the polymerase can only elongate chains in repair-like reactions, it would be unable to transcribe the regions of the RNA involved in the hair-pin structure. This obvious loss of genetic information can be obviated if covalently linked DNA can form hair-pins intramolecularly. Alternatively, *in vivo* initiation may involve other mechanisms. It is possible that small 3'OH-terminated polynucleotides can serve as nucleation points for DNA synthesis, or additional factors may operate to facilitate initiation of DNA on viral RNA without the direct participation of a primer strand. To date, however, no known DNA polymerase can initiate new chains in DNA-directed reactions. If DNA is an intermediate in the replication of viral RNA it is necessary to transcribe this DNA into RNA progeny. Whether this transcription involves a host cell or viral RNA polymerase is unknown. At present, we as well as others (3, 24, 26) have not detected ribonucleotide incorporation with virions or crude extracts of virions under a wide variety of conditions. It is evident that our understanding of the RNA oncogenic viruses would be facilitated by the discovery of the mechanism of this RNA replication.

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ADDENDUM IN PROOF

The work presented in this manuscript was presented in preliminary form at the Federation Meetings in June 1971. After this paper was submitted for publication, evidence for covalent attachment of DNA products to RNA was reported by I. M. Verma, N. L. Meuth, E. Bromfeld, K. F. Manly, and D. Baltimore, *Nature* (London) **233**:131. Also, the authors have since learned that evidence of covalent attachment of RNA to DNA and has been obtained by P. Duesberg and M. Bishop their respective co-workers (*personal communication*).

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