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RNA-Dependent DNA Polymerase Activity of RNA Tumor Viruses

I. Directing Influence of DNA in the Reaction

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The template requirements and deoxyribonucleic acid (DNA) products of the DNA polymerases isolated from Rauscher leukemia and avian myeloblastosis viruses have been examined. All DNA preparations or synthetic polydeoxynucleotides which are active as primers possess a duplex structure containing single-stranded regions with a 3'-hydroxyl terminus. Native DNA and fully single-stranded DNA are inactive; moreover, their activity is not enhanced by sonic oscillation or treatment with micrococcal nuclease, *Neurospora* nuclease, or low levels of deoxyribonuclease I. Poor DNA templates are activated by treatment with exonuclease III, large amounts of deoxyribonuclease I, or an endonuclease isolated from Rauscher viral preparations. In reactions primed with deoxyadenylate-deoxythymidylate copolymer, the product formed is covalently attached to primer strands, indicating that no new strands are initiated. DNA polymerase products formed with exonuclease III- or deoxyribonuclase I-treated DNA are duplex structures. Short single-stranded regions are completely filled in, whereas long single-stranded regions are only partly repaired. DNA preparations containing extensive single-stranded regions are poorly utilized as templates.

The ribonucleic acid (RNA) tumor viruses contain an enzyme capable of transcribing the 60 to 70*S* viral RNA into deoxyribonucleic acid (DNA) (4, 38). The nature of the DNA products formed has been studied extensively with virions rendered partially permeable with nonionic detergents (12, 22). The DNA products are relatively small (6 to 8*S*) and appear to be a mixture of RNA-DNA hybrids, single-stranded DNA, and double-stranded DNA. Kinetic analyses indicate that the initial products formed are RNA-DNA hybrids, whereas free DNA is produced later in the reaction. Duesberg et al. (8) demonstrated that a large excess of DNA products formed with detergent-treated virions annealed to ^{3}H-viral RNA and rendered it quantitatively resistant to ribonuclease. This observation suggests that all unique sequences of the viral RNA are transcribed into DNA. Other studies (39) indicate that a substantial amount of DNA arises from specific regions of the RNA, indicating that all RNA sequences are not copied equally or at the same rate. At present, it is not clear whether the marked heterogeneity of the DNA products reflects transcription of different viral RNA templates (and thus a heterogeneous viral population) or represents selective copying of homogeneous viral RNA within virions.

An important biological role for the viral DNA polymerase has been suggested by evidence indicating the existence of a DNA intermediate in viral replication. This intermediate has been proposed to explain the inhibition of virus production by actinomycin D and cytosine arabinoside (3, 10, 37). Furthermore, Hanafusa et al. (15) obtained a variant of the Rous sarcoma virus (RSVα) which is deficient in DNA polymerase activity; such viral preparations are not infectious nor do they transform chick cells.

The discovery in Spiegelman's laboratory (36) that the viral polymerases in oncornaviruses respond to a variety of polynucleotide primers has been confirmed and extended in other laboratories (5, 9, 23). The high priming efficiency of synthetic homopolymeric duplexes coupled with their availability has greatly facilitated the isolation and characterization of these enzymes. Evidence that both DNA and RNA are used as templates by the viral polymerases has been presented (9).

In the present communication the characteristics of the enzyme from Rauscher leukemia and avian myeloblastosis viruses with DNA and

synthetic polynucleotides as template are reported. These results indicate that the enzyme resembles DNA polymerases isolated from other sources, in particular DNA polymerase II of *Escherichia coli* (19, 20, 26, 41). The viral DNA polymerases do not initiate new chains but incorporate deoxynucleotides onto preexisting chains at 3'-OH ends. For synthesis, the enzyme requires a duplex structure containing a 3'-OH-ended primer strand attached to a template strand with adjacent single-stranded regions to direct nucleotide incorporation. This mechanism was also presented by Baltimore and Smoler (5). A similar conclusion regarding the synthesis of DNA utilizing an RNA template is presented in the accompanying manuscript (21). Some of the results presented here were published in a preliminary report (J. Leis and J. Hurwitz, Fed. Proc. **30**:1153, 1971).

MATERIALS AND METHODS

Reagents. The labeled nucleoside triphosphates were commercial preparations from New England Nuclear Corp., Schwarz BioResearch, Inc., or International Chemical and Nuclear Corp. Nucleoside triphosphates labeled in the γ-phosphate position were purchased from New England Nuclear Corp. or synthesized by the procedure of Glynn and Chappel (13). Calf thymus DNA was obtained from Worthington Biochemical Corp. and purified by deproteinization and alcohol precipitation. Dithiothreitol and dithioerythreitol were obtained from Sigma Chemical Co.

Exonuclease III was purified from *Escherichia coli* by the method of Richardson and Kornberg (31) or obtained as a by-product during the isolation of DNA polymerase II from pol A mutants of *E. coli* (41). Neither enzyme preparation contained detectable endonuclease activity measured with single- or double-stranded DNA (32). The final enzyme preparations were stored at -15 C in 30% glycerol.

Neurospora nuclease was purified by the method of Rabin and Frazer (29) from commercial frozen conidia (Worthington Biochemical Corp.) The final preparation did not liberate detectable acid-soluble nucleotides when incubated with native ^{3}H-λ DNA. These preparations, however, introduce limited numbers of single-strand breaks in native DNA. The latter was measured by the release of acid-soluble ^{3}H-adenosine monophosphate (AMP) from *E. coli* DNA ligase ^{3}H-AMP complex (32).

DNA polymerase I of *E. coli* was "fraction VII" prepared by the method of Jovin et al. (18). DNA ligase from *E. coli* or T4 was prepared as previously described (32). Commercial alkaline phosphatase of *E. coli* (Worthington Biochemical Corp.) was further purified by diethylaminoethyl (DEAE) cellulose chromatography by the procedure of Weiss et al. (40).

^{3}H-labeled λ phage DNA was prepared from lysis-defective lysogens by heat induction of *E. coli* M65 (λCI 857 S7) (14).

The synthetic homopolydeoxynucleotides polydeoxyadenosine (dA), polydeoxythimidine (dT), polydeoxycytidine (dC), and polydeoxyguanosine (dG) were generously provided by Fred Bollum. Polydeoxyinosine (dI)·poly (dC) and poly d(AT) were synthesized either in unprimed or primed reactions with DNA polymerase I. All polynucleotide values reported refer to concentration of nucleotides used.

Methods. Protein was measured by the turbidometric procedure of Bücher (7). When protein solutions contained nonionic detergents, 0.2 ml of 95% ethanol was added. The alcohol increased the solubility of the detergents and prevented them from precipitating on addition of acid.

DNA labeled at 3'-OH ends with ^{3}H-thymidine monophosphate (TMP) was prepared by the limit-reaction catalyzed by DNA polymerase I as previously described (27). DNA labeled at 5'-phosphate termini was prepared by the action of 5'-hydroxylpolynucleotide kinase on native DNA which had been nicked with micrococcal nuclease and treated with alkaline phosphatase at 65 C. This procedure (40) generates 5'-OH and 3'-OH external and internal termini; both types of 5'-OH ends were phosphorylated with an excess of 5'-hydroxylpolynucleotide kinase and γ-^{32}P-adenosine triphosphate (ATP).

Rauscher leukemia virus (RLV) was grown at Pfizer Co., Inc., Maywood, N.J. in mouse cell line JLSV-9 and isolated by isopycnic banding in sucrose gradients (9). Virus was stored in sucrose at 0 C until used; prior to extraction, viral preparations were diluted and concentrated by centrifugation. The authors are indebted to F. Buchett for supplying this virus.

Avian myeloblastosis virus (AMV) was kindly provided by J. Beard, Duke University. The virus was stored at -70 C.

Measurement of polymerase activity. The viral polymerases were measured as follows: reaction mixtures (0.05 ml) contained 5 nmoles of dATP, 1 nmole of ^{3}H-deoxythymidine triphosphate (dTTP) (50 to 300 counts per min per pmole), 2.5 μmoles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.0), 0.5 μmole of $MgCl_2$, 0.25 μmole of dithiothreitol, 2.5 μmoles of KCl, 5 μg of albumin, 5 nmoles of d(AT) copolymer and enzyme. After 30 min at 37 C, the reaction was terminated by the addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.02 ml of denatured salmon sperm DNA (2.6 μmoles/ml), and 5% trichloroacetic acid. After 5 min at 0 C, the acid-insoluble material was collected by filtration on Gelman type E glass fiber filters, dried, and counted in a liquid scintillation counter. One unit of polymerase is defined as that amount of activity which catalyzes the incorporation of 1 nmole of nucleotide under the above conditions in 30 min.

RESULTS

Purification of RLV polymerase activity. Virus was collected by high-speed centrifugation for 75 min at 30,000 rev/min in a Spinco 30 rotor at 4 C. The pellets were resuspended by homogeniza-

tion in 10 ml of a solution containing 0.02 M Tris-hydrochloride (*p*H 8.0), 0.1 M KCl, 5×10^{-3} M dithiothreitol, 5% glycerol, and 0.4% Triton X-100 and then stored at 0 C for 12 hr. The suspension was centrifuged for 30 min at 40,000 rev/min in a Spinco ultracentrifuge, and the supernatant fluid was removed (crude extract 9.8 ml).

Ammonium sulfate fractionation. The crude extract was adjusted to 40% saturation with solid ammonium sulfate (0.226 g/ml), and the mixture was centrifuged for 15 min at 40,000 rev/min. The precipitate floated at the surface due to the presence of the Triton X-100. The clear supernatant fluid was carefully removed with a Pasteur pipette, avoiding loss of any floating material. The precipitate was dissolved with 4 ml of 0.02 M Tris-hydrochloride (*p*H 8.0), 0.01 M 2-mercaptoethanol, 10^{-4} M ethylenediaminetetraacetate (EDTA), and 5% glycerol (buffer A). The ammonium sulfate fraction was dialyzed for 3.5 hr against two changes of 500 ml of buffer A. The salt concentration of the dialyzed fraction was reduced below 0.03 M as measured with a Barnsted Purity Conductivity Meter.

Phosphocellulose chromatography. A column of P-11 phosphocellulose (2.5 by 10 cm) which had been washed with acid, alkali, and water was equilibrated with buffer A. The dialyzed fraction described above was adsorbed to the phosphocellulose which was then washed with 20 ml of buffer A. The enzyme activity was eluted from the column with a linear gradient of the following concentration and volume: 300 ml of buffer A and 300 ml of buffer A containing 0.5 M KCl. The enzyme activity eluted at 0.22 M KCl, and the eluates containing enzyme activity were pooled and concentrated rapidly by dialysis against buffer A containing 30% polyethylene glycol in place of glycerol.

Glycerol gradient centrifugation. A sample (0.2 ml) of the concentrated enzyme solution was layered onto a glycerol gradient (10 to 35%) in 0.05 M NaCl, 0.02 M Tris-hydrochloride (*p*H 7.9), 2×10^{-4} M EDTA, and 5×10^{-3} M 2-mercaptoethanol and centrifuged at 55,000 rev/min for 18 hr in an SW65 rotor. Fractions were collected after piercing a hole in the bottom of the tube. Virtually all of the activity was isolated in a single peak corresponding to a molecular weight of 90,000 (relative to a catalase marker).

A summary of the yield and purification is presented in Table 1. The enzyme activity was purified a minimum of 75-fold by the above procedure with a 50% yield. The degree of purification achieved after glycerol gradient centrifugation has not been assessed because of the lack of detectable protein and our reluctance to use large amounts of a limited supply of enzyme for protein analysis.

TABLE 1. *Purification of polymerase from Rauscher leukemia virus*[a]

Purification step	Total units	Specific activity (μmoles/mg/30 min)	Total protein (mg)
Crude extract	750	0.07	11.4
Ammonium sulfate	972	0.10	9.7
Phosphocellulose eluate	581	5.3	0.11
Glycerol gradient[b]	490	>5.3	

[a] This isolation was carried out with 2.23×10^{13} virus particles, as measured by electron microscopic count.

[b] This step was carried out with only a small fraction of the phosphocellulose eluate. The total units recovered were calculated assuming that the entire phosphocellulose fraction was used.

The enzyme preparation stored as a crude extract is relatively stable, showing no loss of activity over a period of 3 months; similar stability was observed with the ammonium sulfate fraction. In contrast to this, the phosphocellulose eluate lost 50% of its activity after 72 hr; this loss could be prevented by dialysis against 50% glycerol or by concentration against polyethylene glycol. Such preparations retained 30 to 50% of their activity after 1 month of storage at −10 C. A similar gradual loss of activity was observed with glycerol gradient fractions.

The enzyme preparation obtained after phosphocellulose chromatography was free of detectable endonuclease [measured by alkaline sucrose gradient centrifugation (32) of native ^{3}H-λ DNA] or exonuclease activity on native ^{3}H-λ DNA. The endonuclease activity which attacked DNA was detected in low salt fractions of the phosphocellulose column and was further purified (20-fold) by chromatography on DEAE cellulose by stepwise salt elution. The nuclease eluted with 0.5 M KCl from DEAE cellulose. The endonuclease activity attacked all DNA species examined. Further discussion of this activity is presented below.

Extracts of RLV contained ribonuclease activity capable of attacking either ^{3}H-labeled RLV RNA or ^{3}H-polyuridylate. More than 99% of the ribonuclease activity of the crude extract was removed during phosphocellulose fractionation. No detectable acid-soluble material was generated when the glycerol gradient enzyme was incubated with ^{3}H-labeled RLV RNA nor was there significant change in the sedimentation profile of this RNA. In contrast to this, significant

acid-soluble material was detected after incubation with ^{3}H-polyuridylic acid (U). This difference may reflect the difference in structure of the RNA preparations. As presented in the accompanying paper (21), demonstrable ribonuclease activity was noted in these enzyme preparations (on RLV RNA) only after destruction of the secondary structure of this RNA.

Purification of AMV DNA polymerase. Unless otherwise specified, all operations were carried out at 4 C. The AMV, suspended in plasma, [4 g of virus as measured by adenosine triphosphatase activity (6)] was concentrated by centrifugation at 105,000 × *g* for 30 min in a Spinco no. 40 rotor. The pellet was suspended by homogenization with a loose-fitting Teflon homogenizer in 20 ml of 0.4% Triton X-100, 40 mM Tris-hydrochloride (*p*H 8.0), 0.1 M NaCl, 5 mM dithioerythreitol, and 10% glycerol (buffer B). After 10 min, the suspension was centrifuged at 105,000 × *g* for 15 min, and the supernatant fluid was removed. The residue was extracted three more times with 20 ml of buffer B, and all of the supernatant fluids were combined (crude extract). Approximately 90 to 95% of the polymerase activity was solubilized by this treatment.

The crude extract was precipitated with ammonium sulfate (2.6 g/10 ml), and the insoluble material was collected by centrifugation at 105,000 × *g* for 10 min. The precipitate was dissolved in a final volume of 14 ml of buffer B without detergent. The ammonium sulfate fraction was dialyzed for 2 hr against five changes of 300 ml each of 20 mM Tris-hydrochloride (*p*H 8.4), 0.1 mM EDTA, 5 mM mercaptoethanol, and 10% glycerol (buffer C), after which time the salt concentration was less than 0.03 M ammonium sulfate as determined by conductivity measurement. The dialysate was applied to a 200-ml Whatman P-11 phosphocellulose column equilibrated with buffer C. The column was eluted with a 1,200-ml linear ammonium sulfate salt gradient from 0 to 0.4 M in buffer C. Ten-milliliter fractions were collected by drop counting, and samples were assayed for polymerase activity as described below. Two peaks of polymerase activity were detected eluting from the phosphocellulose column at 0.05 and 0.15 M ammonium sulfate, respectively. The first peak represented only 5% of the total polymerase activity and was not studied further. The second peak, representing 95% of the total polymerase activity, was pooled and coprecipitated with added serum albumin (0.3 mg/ml) by the addition of ammonium sulfate (47 g/100 ml). The precipitate was collected by centrifugation and dissolved in 1.8 ml of buffer C, samples were layered on 5 ml of 20 to 40% linear glycerol gradients containing 20 mM Tris-hydrochloride (*p*H 7.9), 10 mM 2-mercaptoethanol, 50 mM NaCl, and 0.1 mM EDTA. The gradients were centrifuged 21 hr at 55,000 rev/min in a Spinco SW65 rotor. The tubes were pierced at the bottom, and 15-drop fractions were collected. Tubes containing polymerase activity were used directly for experiments cited below.

The AMV DNA polymerase was purified approximately 80-fold over the activity detected in the ammonium sulfate fraction (Table 2).

Crude extracts of the AMV contained a potent nondialyzable inhibitor of the DNA polymerase which almost completely masked the activity present; routinely, there has been a marked increase (>20-fold) in activity after ammonium sulfate fractionation provided that enzyme activity at this stage is measured after extensive dilution. The inhibitor is quantitatively removed from the polymerase preparations during chromatography on phosphocellulose. At the present time, the nature of this inhibitor is unknown. After phosphocellulose chromatography, the polymerase preparations were free of deoxyribonuclease activity but contained small amounts of ribonuclease. Passage of the polymerase through a glycerol gradient removed most of the remaining ribonuclease as measured by acid solubilization of ^{3}H-poly (U) (21). Ribonuclease activity, however, was still detected in glycerol gradient fractions when assayed with ^{3}H-labeled RLV RNA followed by sedimentation in a formaldehyde sucrose gradient. Enzyme fractions virtually free of detectable ribonuclease activity measured by the more sensitive centrifugation assay have been prepared by passage of the polymerase through a second phosphocellulose column at *p*H 7.0 (21).

TABLE 2. *Purification of DNA polymerase from Avian myeloblastosis virus*

Purification step	Total units	Specific activity (μmoles/mg/30 min)	Total protein (mg)
Crude extract	240	0.0013	189
Ammonium sulfate	5,262	0.04	130
Phosphocellulose eluate—peak I (0.05 M)	191	1.35	0.14
Phosphocellulose eluate—peak II (0.15 M)	2,490	3.11	0.08
Glycerol gradient[a]	1,818	>3.11	

[a] This step has been carried out with only a small fraction of the phosphocellulose 0.15 M eluate. The total units recovered are calculated assuming that the entire phosphocellulose fraction was used.

The molecular weight of the AMV polymerase was estimated to be 160,000 by glycerol gradient centrifugation relative to a serum albumin marker. The polymerase could be stored frozen at −10 C in the presence of at least 15 to 20% glycerol without appreciable loss in enzyme activity over several days. After this period, a gradual decrease in enzyme activity was noted which could be recovered by concentrating enzyme solutions by pressure dialysis.

The properties of both viral polymerases are similar in response to *p*H, SH inhibitors, and affinity of various polymers. With the purified RLV DNA polymerase assayed with d(AT) copolymer, the K_m for dATP was 1.5×10^{-5} M; the K_m for dTTP was 2.6×10^{-5} M, whereas the K_m for Mg^{2+} was 2.5×10^{-3} M. KCl at 5×10^{-3} M stimulated the reaction approximately 30% compared to reaction mixtures lacking this salt. The K_m for thymus DNA (activated with exonuclease III) was 1.1×10^{-5} M. The RLV polymerase was highly sensitive to reagents which react with SH groups. Thus *p*-hydroxymercuribenzoate at 2×10^{-5} M inhibited the reaction 96%, whereas *N*-ethylmaleimide at 2×10^{-3} M inhibited the reaction 82%. These inhibition studies were carried out in reaction mixtures lacking sulfhydryl reagents.

Priming of polymerase reaction with synthetic polydeoxynucleotide. The RLV and AMV polymerases show similar activity with synthetic polydeoxynucleotides as templates. As shown in Table 3, reactions primed with d(AT) copolymer resulted in the incorporation of equal amounts of dTMP and dAMP. Nucleotide incorporation with d(AT) copolymer as primer was dependent on the presence of both dATP and dTTP; the omission of either nucleotide resulted in virtually no incorporation of the other nucleotide. With poly(dA)·poly(dT) as primer, both nucleotides were incorporated, although not equally or interdependently under the conditions employed. In contrast to incorporation of both deoxynucleotides observed with the above polymers, priming with poly (A)·poly (dT) resulted in only dTMP incorporation. Spiegelman et al. (36) and Baltimore and Smoler (5) have reported similar observations. In further agreement with these workers, we found that poly (dA)·poly (U) and poly (A)·poly (U) poorly support nucleotide incorporation. These templates were measured for their priming ability in the presence of Mg^{2+}; as reported by Scolnick et al. (34), the presence of Mn^{2+} increased the efficiency of a template such as poly (A)·poly (U). In the absence of complementary homopolymers, there was no significant incorporation under the conditions used.

TABLE 3. *Priming of viral polymerases with adenosine (A), uridine (U), and thymidine (T) containing polynucleotides*[a]

Polynucleotide primer added	Incorporation (pmoles/30 min) of			
	Deoxythymidine monophosphate		Deoxyadenosine monophosphate	
	Rauscher polymerase	Avian polymerase	Rauscher polymerase	Avian polymerase
Poly d(AT)	41.2	34.3	48	33
Poly (dA)·poly (dT)	27.3	25.5	44.3	31
Poly (A)·poly (dT)	202	222	0.3	0.3
Poly (dA)·poly (U)	0.3	0.2	2.9	0.3
Poly (A)·poly (U)	5.6	1.4	0.3	0.3
Poly (dA) or poly (dT) or poly (A), or poly (U)	0.3	—[b]	0.3	—[b]

[a] Reaction mixture (0.05 ml) containing 0.05 M tris (hydroxymethyl)aminomethane-hydrochloride (*p*H 8.0), 10^{-2} M $MgCl_2$, 5×10^{-3} M dithiothreitol, 5×10^{-3} M KCl, 2×10^{-5} M ^{3}H-deoxythymidine triphosphate (300 counts per min per pmole), 3×10^{-5} M α-^{32}P-deoxyadenosine triphosphate (860 counts per min per pmole), and, where indicated, 2.7×10^{-5} M d(AT) copolymer, 4×10^{-5} M poly (dA)·poly (dT), 1.6×10^{-5} M poly (dT) + 6.8×10^{-5} M poly (A), 6.8×10^{-5} M poly (A) + 4×10^{-5} M poly (U), and 0.02 μg of Rauscher polymerase (phosphocellulose fraction) or 0.11 μg of avian polymerase (phosphocellulose fraction) was incubated for 30 min at 38 C. The reaction was halted by the addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.025 ml of denatured salmon sperm DNA (2.6 μmoles per ml), and 5% trichloroacetic acid. After 5 min at 0 C, the mixture was filtered through Gelman type E glass fiber filters, dried, and counted in toluene-1,4-bis-2-(5, phenyloxazolyl)-benzene under double-labeling counting conditions.

[b] Indicates that measurements with the avian system were not carried out.

Suitably altered thymus DNA (*see below*) supported the incorporation of deoxyguanosine monophosphate (dGMP) and deoxycytidine monophosphate (dCMP) at equal rates (Table 4). With GC homopolymers, it was found that there was a marked selection for one nucleotide over the other. With poly (dI)·poly (dC) as primer, only dGMP incorporation was demonstrable; similar observations were made with poly (dG)·poly (dC). However, with poly (I)·poly (dC), only dCMP incorporation was noted; with poly (G)·poly (dC), virtually no nucleotide incorporation was noted. These results are in

TABLE 4. *Priming of Rauscher polymerase with synthetic guanosine (G), inosine (I), and cytidine (C) containing polynucleotides*[a]

Polynucleotide primer added	Incorporation (pmoles/30 min) of	
	Deoxyguanosine monophosphate	Deoxycytidine monophosphate
Thymus DNA	5.5	5.7
Poly (dI)·poly (dC)	32	0.32
Poly (dG)·poly (dC)	42	0.8
Poly (I)·poly (dC)	0.32	28
Poly (I)·poly (C)	0.13	2.1
Poly (dG)·poly (C)	1.6	0.1
Poly (G)·poly (dC)	0.5	0.1
Poly (dC) or poly (C) or poly (dG) or poly (I)	<1.0	<1.0

[a] Reaction mixtures were as described in Table 3 with the exception that α-^{32}P-deoxyguanosine triphosphate (350 counts per min per pmole) and ^{3}H-deoxycytidine triphosphate (112 counts per min per pmole) were used and the following polymers were added: poly (dI)·poly (dC), 4×10^{-5} M; poly (dC), 3.2×10^{-5} M, + poly (I), 7.6×10^{-5} M; poly (dG), 2.4×10^{-5} M, + poly (dC), 3.2×10^{-5} M; poly (G), 4×10^{-5} M, + poly (dC), 3.3×10^{-5} M; poly (I)·poly (C), 5.6×10^{-5} M; poly (C), 8×10^{-5} M, + poly (dG), 2.4×10^{-5} M. All other conditions were the same. The results presented here were those obtained with the Rauscher polymerase. Identical results were obtained with the avian polymerase. The poly (I)·poly (C) was prepared as follows: 1 ml of poly (C), 21.3 μmoles, heated to 70 C + 1 ml of poly (I), 22.5 μmoles, heated to 70 C in 0.15 M NaCl plus 6×10^{-3} M potassium phosphate buffer (*p*H 7) were mixed at 70 C with constant stirring for 10 min and then slowly cooled to 45 C. The mixture was then slowly cooled to room temperature. This treatment resulted in a hypochromic effect of 29%. Such solutions were stored at 4 C to prevent irreversible precipitation which occurs upon freezing and thawing. Reaction mixtures which contained thymus DNA also included endonuclease isolated from Rauscher viral extracts as well as deoxyadenosine triphosphate and deoxythymidine triphosphate (5×10^{-5} M each).

contrast to the reported marked preference for dGMP incorporation reported by Spiegelman et al. (36) with these templates. We have also found that poly (I)·poly (C) and poly (dC) are poor templates. These studies do not support the contention that the priming reactions observed with the avian polymerase (or Rauscher enzyme) resemble the reactions catalyzed by Qβ replicase or DNA-dependent RNA polymerases (36).

Priming of polymerases with DNA. The presence of DNA-dependent DNA polymerase in RNA tumor viruses was first demonstrated by Spiegelman et al. (35). These workers showed that a variety of exogenously supplied DNA preparations supported deoxynucleotide incorporation; they also noted a marked preference for double-stranded DNA and suggested that this feature distinguished the viral polymerase from previously described DNA-dependent DNA polymerases.

We reinvestigated this problem and found, as they reported, that the polymerases exhibit a marked preference for native DNA. However, as will be discussed further, this preference is due to the requirement for a 3'-OH-containing primer strand hydrogen bonded to an overlapping template strand. This characteristic is true for all DNA polymerase activities so far studied. The only exception to this general requirement in primed deoxynucleotide incorporation is the terminal deoxynucleotidyl transfer enzyme studied by Bollum and co-workers (17).

Crude extracts and ammonium sulfate fractions of Rauscher and avian polymerases used native DNA (λ, thymus, T7, etc.) as primers in supporting deoxynucleotide incorporation. The ammonium sulfate fraction obtained from RLV utilized poly (I)·poly (dC) and thymus DNA with almost equal facility (Table 5). Since reactions primed with poly (I)·poly (dC) lead to dCMP incorporation, whereas reactions primed

TABLE 5. *Comparison of priming by poly inosine (I)·poly deoxycytidine (dC) versus native DNA with different enzyme fractions*[a]

Enzyme fraction	DNA substrate added	^{3}H-dCMP incorporation (pmoles/30 min)
Ammonium sulfate	Poly (I)·poly (dC)	105
	Thymus DNA	26
	None	<1
Phosphocellulose eluate	Poly (I)·poly (dC)	124
	Thymus DNA	<1
	None	<1

[a] Reaction mixtures (0.05 ml) contained: 1.5 nmoles of ^{3}H-deoxycytidine triphosphate, 5 nmoles each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, dCTP with thymus DNA; 2.5 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 8.0), 0.5 μmole of $MgCl_2$, 0.25 μmole of dithiothreitol, 0.25 μmole of KCl, 5 μg of albumin, 3.8 nmoles of poly (I) + 0.8 nmole of poly (dC) or 18 nmoles of calf thymus DNA (as indicated), ammonium sulfate fraction (7.8 μg) or phosphocellulose eluate (0.032 μg). These fractions were obtained from the Rauscher virus. All other conditions used in this reaction were as previously described.

with thymus DNA result in incorporation of all four deoxynucleotides, the activity observed with these primers is identical. After chromatography of the ammonium sulfate fraction on phosphocellulose, there was no detectable activity with native DNA as primer; the activity with poly (I)·poly (dC) was unchanged. These results suggested that a factor had been inactivated or separated from the polymerase during purification. The activity observed with native DNA was fully reconstituted when fractions containing polymerase activity were mixed with the 0.05 M KCl salt eluates obtained during phosphocellulose chromatography (Table 6). This activity was purified further approximately 20-fold by chromatography on DEAE cellulose by using stimulation of synthesis in the presence of excess purified polymerase as the assay. The stimulatory activity was inactivated by heating at elevated temperatures (80 C for 3 min or 100 C for 2 min). This fraction, containing the stimulatory activity, catalyzed a rapid breakdown of ^{14}C-labeled T7-DNA, reducing its size measured in alkaline or neutral sucrose gradients without producing acid-soluble material. We have concluded that this stimulating factor which altered the poor priming efficiency of native DNA is most likely an endonuclease. This activity can be replaced by pancreatic deoxyribonuclease or by exonuclease III, as discussed below.

Alterations of templates and their influence on priming efficiency. The structural modification required to activate native DNA as template was studied. Treatment of native thymus DNA with exonuclease III or the endonuclease activity associated with RLV or with relatively large amounts of pancreatic deoxyribonuclease (Table 7) activated it as a template. The following treatments were without effect: sonic oscillation, incubation with *Neurospora* nuclease or micrococcal nuclease, heat denaturation, and introduction of single-strand breaks with pancreatic deoxyribonuclease.

TABLE 6. *Isolation of factor from Rauscher virions which activates native DNA*[a]

Enzyme fractions used	Activity with (pmoles of ^{3}H-dCMP incorporated/30 min)	
	Poly (I)·poly (dC)	Native thymus DNA
1. Ammonium sulfate	29.2	8.5
2. 0.05 M KCl phosphocellulose eluate	0.35	0.7
3. Polymerase fraction (0.22 M KCl)	40.8	0.3
4. KCl phosphocellulose eluate plus polymerase fraction (no. 2 + no. 3)	38.7	11.6

[a] Reaction mixtures were as described in Table 5 with the exception that the concentration of protein used was reduced, e.g., ammonium sulfate fraction (2.93 μg) or phosphocellulose fraction (0.014 μg). The 0.05 M salt eluate was obtained from the phosphocellulose fractionation procedure described above (*see* Table 1). In this assay, 0.095 μg of protein of this low-salt eluate was employed.

The low levels of deoxyribonuclease employed (Table 7) optimally activate calf thymus DNA as a template for DNA polymerase I (2). The single-strand breaks introduced by this procedure do not activate DNA as a primer for the RLV or AMV polymerases (*data not shown*). Since DNA polymerase I utilizes these single strand breaks by "nick translocation" due to the presence of the 5′→3′ exonuclease (18), these observations suggest that the RLV and AMV polymerases lack such an exonuclease activity. This interpretation was substantiated by the following experiments. DNA containing 5-^{32}P-labeled ends was not attacked by Rauscher polymerase preparations. In addition, limit addition products formed with DNA polymerase I containing ^{3}H-dTMP at the 3′-OH end were also not attacked. These results indicate that the Rauscher polymerase is devoid of both the 5′→3′ and 3′→5′ exonuclease activities with native DNA. [In these experiments, 5.8 nmoles of λ DNA (sonically treated) containing 1.43 pmoles of ^{32}P (1,100 counts per min) as termini was incubated with 0.21 unit of Rauscher polymerase for 30 min at 38 C. No detectable acid-soluble ^{32}P was formed. These preparations contained 0.74 pmole of ^{32}P in internal 5′-phosphate ends as measured directly with DNA ligase (40). In experiments carried out with 3′-hydroxyl-labeled DNA, DNA (12.3 nmoles) containing 4.1×10^{3} counts per min of ^{3}H-TMP at 3′-OH ends was incubated with 0.17 unit of Rauscher polymerase for 30 min at 38 C. No detectable acid-soluble ^{3}H was detected.] These experiments were not carried out with the avian polymerase preparation.

The quantitative relationship between primer and template strands was investigated with poly (A)·poly (dT) (Table 8). Either polymer alone did not support deoxynucleotide incorporation (*data not shown*). Under conditions in which the poly (A) concentration was constant, the rate of dTMP incorporation increased and then decreased as the poly (dT) concentration was increased. The elevated dTMP incorporation noted is most likely due to the increase in 3′-OH terminated strands in duplex structures; the decrease in incorporation can be explained by the titration of the poly (A) by the poly (dT) resulting

TABLE 7. *Template requirements of Rauscher DNA polymerase*

Treatment of native DNA	Incorporation of ^{3}H-dCMP (pmoles/30 min)
None	0.4
Rauscher endonuclease[a]	8.7
Sonic treatment[b]	0.2
Neurospora nuclease[c]	<0.1
Pancreatic deoxyribonuclease (0.18 ng)[d]	0.3
Pancreatic deoxyribonuclease (0.18 μg)	11.4
Micrococcal nuclease (0.015–1.5 μg[e]	0.4–0.1
Exonuclease III[f]	6.9
None—heated at 100 C for 3 min[g]	<0.1
Rauscher endonuclease,[a] followed by heating at 100 C for 3 min	0.3
Pancreatic deoxyribonuclease,[d] followed by heating at 100 C for 3 min.	1.1

[a] Reaction mixtures (0.5 ml) containing 50 μmoles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, (*p*H 8.0), 5 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 10 μmoles of KCl, 0.15 mg of albumin, 487 nmoles of calf thymus DNA, and 0.42 μg of Rauscher endonuclease isolated after diethylaminoethyl cellulose chromatography were incubated for 30 min at 38 C. After this period the reaction mixture was heated to 65 C for 10 min to inactivate the endonuclease.

[b] Calf thymus DNA (0.97 μmoles/ml) in 0.05 M NaCl, 0.01 M Tris-hydrochloride (*p*H 8.0), 0.005 M ethylenediaminetetraacetic acid (EDTA) was sonically treated for 45 sec at 20 kc in the Branson sonifier.

[c] Reaction mixtures (0.25 ml) containing 10 μmoles of Tris-hydrochloride (*p*H 7.5), 1 μmole of $MgCl_2$, 448 nmoles of calf thymus DNA, and 0.4 unit of *Neurospora* nuclease were incubated for 60 min. Reaction mixtures were heated at 65 C for 10 min to inactivate the nuclease.

[d] Reaction mixtures were as described for the Rauscher endonuclease with the substitution of pancreatic deoxyribonuclease.

[e] Reaction mixture (0.25 ml) contained 15 μmoles of Tris-hydrochloride (*p*H 8.6), 224 nmoles of calf thymus DNA, 0.25 μmole of $CaCl_2$, and the indicated amount of micrococcal nuclease. After 30 min at 37 C, reaction mixtures were treated with 5 μmoles of EDTA and 5 μmoles of 2-mercaptoethanol and heated at 65 C for 15 min to inactivate the endonuclease.

[f] Reaction mixtures (0.25 ml) containing 15 μmoles of Tris-hydrochloride (*p*H 8.0), 1 μmole of $MgCl_2$, 5 μmoles of dithiothreitol, 448 nmoles of calf thymus DNA, and 12 units of exonuclease III were incubated for 30 min at 37 C and then heated at 65 C for 10 min.

[g] In all experiments where heat treatment occurred, tubes were immersed in ice immediately after heating. All other additions were as described in the legend to Table 5 with Rauscher DNA polymerase (01023 μg of protein) and approximately 10 nmoles of DNA.

TABLE 8. *Influence of ratio of primer to template strand on nucleotide incorporation*[a]

Additions (in nmoles)	dTMP incorporation (pmoles/30 min)
Poly (A) (1.7)·poly (dT) (0.05)	70
Poly (A) (1.7)·poly (dT) (0.10)	81
Poly (A) (1.7)·poly (dT) (0.25)	100
Poly (A) (1.7)·poly (dT) (0.50)	91
Poly (A) (1.7)·poly (dT) (1.0)	60
Poly (A) (1.7)·poly (dT) (2.5)	42
Poly (A) (1.7)·poly (dT) (5.0)	5.5

[a] Reaction mixtures (0.05 ml) contained the indicated amount of nucleotides as poly adenosine (A) and poly deoxythymidine (dT) with 1 nmole of ^{3}H-deoxythymidine triphosphate (308 counts per min per pmole) and 1 nmole of α-^{32}P-deoxyadenosine phosphate (800 counts per min per pmole). All other conditions were as described in the legend to Table 3 with Rauscher polymerase (phosphocellulose fraction). Under the conditions employed, deoxythymidine monophosphate (dTMP) incorporation was proportional to enzyme concentration used (0.02 and 0.1 μg of protein). No detectable ^{32}P incorporation was observed under the conditions described above.

in the loss of template strands essential for directing incorporation. It is also possible that triple-stranded structures are generated which inhibit this system. Similar observations were found with poly (I)·poly (dC) templates.

Nature of the primer and product. Exonuclease III treatment or extensive nuclease action activate native DNA templates. These observations suggest that the polymerases catalyze incorporation of deoxynucleotides at the 3′-OH end of one strand and utilize the single-stranded regions of the complementary strand as template. If this model is correct, structures resembling those produced by the action of exonuclease III (Fig. 1) would be active templates. Also, since *Neurospora* nuclease specifically attacks single-stranded regions in DNA as well as RNA, such treatment should destroy the priming efficiency of any active DNA (or RNA). The duplex structure generated by the "repair" action of the polymerase should also be resistant to the digestion by the *Neurospora* nuclease.

The addition of the *Neurospora* nuclease to reaction mixtures containing DNA activated by the action of Rauscher endonuclease, pancreatic deoxyribonuclease, or exonuclease III significantly reduced deoxynucleotide incorporation (Table 9). Polymers, such as poly (dI)·poly (dC) which specifically lead to dGMP incorporation were also inactivated by the action of *Neurospora* nuclease. Thus, all active substrates possess a primer-template structure, and the template must

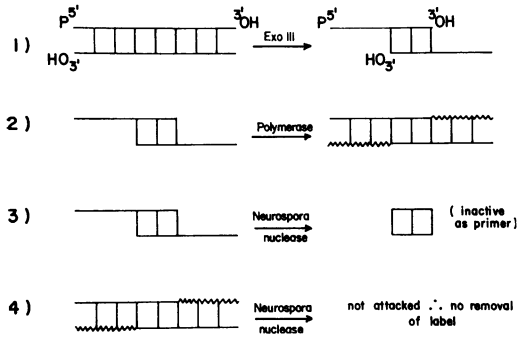

FIG. 1. *Proposed action of polymerase and Neurospora nuclease on DNA primers and products. The structures proposed above are those expected by the action and specificity of the enzymes indicated.*

TABLE 9. *Influence of Neurospora nuclease on priming activity of DNA templates*[a]

Expt	Additions	dTMP incorporation (pmoles/30 min)
1	Complete system (deoxyribonuclease I-treated)	44.4
	Complete system + *Neurospora* nuclease	6.2
	Complete system (DNA exonuclease III-treated)	29.4
	Complete system (exonuclease III-treated) + *Neurospora* nuclease	1.9
2[b]	Poly (dI)·poly (dC)	180
	Poly (dI)·poly (dC) + *Neurospora* nuclease	10

[a] Reaction mixtures (0.05 ml) were as described in the legend to Table 5 with DNA treated as described in the legend to Table 7. In experiment 1, 10 nmoles of DNA was present while 0.04 unit of *Neurospora* nuclease was added where indicated. In experiment 2, 2 nmoles of poly deoxyinosine (dI)·poly deoxycytidine (dC) was added with α-^{32}P-deoxyguanosine triphosphate (1.5 nmoles); all other additions were as described previously.

[b] Values in experiment 2 represent picomoles of deoxyguanosine monophosphate incorporated per 30 min.

contain single-strand regions which are susceptible to *Neurospora* nuclease.

The expected product of repair synthesis is a duplex structure resistant to *Neurospora* nuclease. Results in accord with this mechanism are presented in Table 10. As shown, the products formed in the polymerase reaction are resistant to the action of *Neurospora* nuclease but are completely susceptible after heat denaturation.

TABLE 10. *Susceptibility of product to Neurospora nuclease*[a]

DNA primer added	Heat treatment	Insoluble DNA (pmoles)
Escherichia coli treated with exonuclease III	55 or 100 C	35
	55 C + nuclease	28
	100 C + nuclease	0.3
Poly (dI)·poly (dC)	55 or 100 C	301
	55 C + nuclease	286
	100 C + nuclease	196

[a] Products were formed with Rauscher DNA polymerase as described in the legend to Table 5. After 30 min of incubation, the products were either heated at 55 C for 10 min to inactivate the polymerase or heated at 100 C for 5 min to denature the DNA product as well. Portions were added to reaction mixtures (0.1 ml) described in the legend to Table 9 for *Neurospora* nuclease action and treated with 0.08 unit of *Neurospora* nuclease for 30 min at 37 C. The amount of acid-insoluble radioactivity was measured as previously described.

When products formed in reactions primed with poly(dI)·poly (dC) were exposed to the same treatment, denaturation hardly increased the susceptibility of the product to *Neurospora* nuclease action. This is expected of a product which can undergo reversible denaturation-renaturation.

Direct evidence for the incorporation of nucleotides at the 3'-OH end of exonuclease III-treated DNA has been obtained. With ^{3}H-λ DNA, it was found that incorporated deoxynucleotides (37 pmoles of ^{32}P-dGMP) were quantitatively removed by the action of exonuclease III, whereas fewer than 4 pmoles of ^{3}H-λ DNA were rendered acid-soluble during the reaction.

Covalent linkage of newly synthesized DNA to primer DNA. The data presented above suggest that there is incorporation of deoxynucleotides at the 3'-OH ends of duplex structures rather than the initiation of new DNA chains. Proof of this mechanism was obtained by demonstrating covalent attachment of the product to the primer DNA. In these experiments, ^{3}H-d(AT)copolymer was used as a primer, and synthesis was carried out with ^{32}P-dATP and unlabeled TTP. After 15% synthesis of ^{32}P-d(AT) copolymer, the sedimentation profile of the ^{3}H-d(AT) copolymer primer and the ^{32}P-d(AT) copolymer product was analyzed in alkaline sucrose gradients (Fig. 2A). Both ^{32}P- and ^{3}H-labeled polymers cosedimented almost identically; the peak of ^{32}P-d(AT) product, however, sedimented slightly slower than the

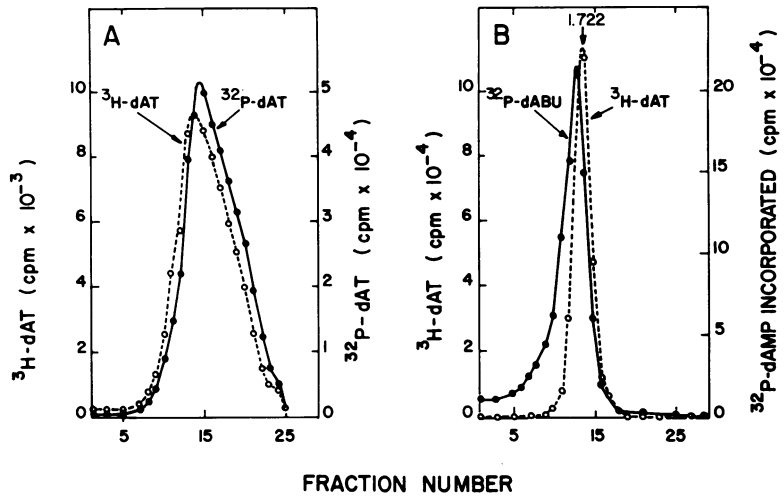

FIG. 2 *(A) Sedimentation profile of ^{3}H-d(AT) copolymer primer and ^{32}P-d(AT) product in alkaline sucrose. Reaction mixtures (0.05 ml) were as described in the legend to Table 3 with 2.0 nmoles of ^{3}H-d(AT) copolymer (4.2 × 10^7 counts per min per μmole), 2 nmoles of dTTP, 1 nmole of α-^{32}P-dATP (2,000 counts per min per pmole), and 0.54 unit of Rauscher polymerase. After 30 min at 38 C, approximately 15% synthesis of ^{32}P-d(AT) copolymer was detected. Reaction mixtures were treated with 0.02 ml of 1 M EDTA, 0.13 ml of 2 M NaOH; the entire reaction mixture was layered on to a 5 to 20% sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl, and 0.005 M EDTA and centrifuged for 120 min at 50,000 rev/min in SW50 rotor at 4 C. Fractions were collected, and the acid-insoluble ^{3}H and ^{32}P were measured. The recovery of input ^{3}H and ^{32}P was greater than 90% in both cases. The sedimentation profile of ^{3}H-d(AT) copolymer presented above was identical to the sedimentation profile observed with copolymer unexposed to Rauscher polymerase preparations in keeping with the absence of endonuclease activity in these fractions. (B) Equilibrium sedimentation in CsCl solution of ^{3}H d(AT) primer and ^{32}P-d(ABu)-containing product. Reaction mixtures (0.1 ml) contained 21 nmoles of ^{3}H-d(AT), 3 nmoles of dBrUTP, 2.5 nmoles of α-^{32}P-dATP (1,850 counts per min per pmole), and 0.40 unit of Rauscher polymerase (phosphocellulose eluate). All other conditions were the same as described before. Incubation was for 30 min, at which time 650 pmoles of ^{32}P had been incorporated into an acid-insoluble form. Reaction mixtures lacking dBrUTP incorporated less than 15 pmoles of ^{32}P into an acid-insoluble form. Reaction mixtures were treated with 0.02 ml of 1 M EDTA, 0.2 ml of 2 M NaOH, 1.88 ml of water, and 2.73 g of CsCl and then centrifuged at 40,000 rev/min at 25 C in polyallomer tubes for 44 hr. The density was measured on samples collected after piercing the bottom of the tubes and the amount of acid-insoluble radioactivity was determined. The banding density of the ^{3}H-d(AT) corresponds to 1.722 and the recovery of ^{32}P in acid-insoluble material was 91%. The experiments described above were performed with the purified avian polymerase PI fraction with identical results.*

^{3}H-d(AT) copolymer primer. This difference would be expected if preferential utilization of smaller species of ^{3}H-d(AT) copolymer as primer occurred.

Further evidence supporting covalent linkage between the primer and product was obtained. For this purpose, reactions carried out with α-^{32}P-dATP and deoxybromouridine triphosphate (dBrUTP) and ^{3}H-d(AT) copolymer as primer were analyzed by banding in alkaline CsCl solution (Fig. 2B). If new chains are initiated during DNA synthesis, ^{32}P-labeled product would be found at the density of heavy deoxyadenylate-deoxybromouridylate [d(ABu)] copolymer. If, however, dBuMP and ^{32}P-dAMP were covalently attached to the ^{3}H-d(AT) copolymer, the banding density would depend upon the contribution of primer material to product formed. As shown, under conditions of only 3 to 5% synthesis of ^{32}P-product (relative to input primer), both the ^{32}P-product and the ^{3}H-primer band at a light density. There is no indication of any completely dense material (expected near the bottom of the gradient). [Similar experiments with d(ABu) copolymer synthesis and banding in alkaline CsCl solution have been performed with DNA polymerases I and II (41). These experiments were carried out with the identical ^{3}H-d(AT) copolymer employed in the above experiment. Products with DNA polymerase II showed no material banding at the fully density-labeled positions, whereas products formed with DNA polymerase I contain some fully density-labeled DNA probably due to nuclease action (5′ → 3′) at the light 5′ end of the d(AT) copolymer chain.]

Extent of repair synthesis in the polymerase system. The mechanism of synthesis outlined

above suggests that the tumor virus polymerases catalyze only a repair synthesis. We therefore studied the extent of single-stranded regions of exonuclease III-treated DNA which the viral polymerase could repair. For this purpose, ^{3}H-λ DNA was sonically treated to a size corresponding to a molecular weight of 10^6 and then treated with exonuclease III; the extent of nucleotides removed was measured. Preparations in which up to 50 nucleotides had been removed were quantitatively repaired (Fig. 3). At the time indicated (30 min), more enzyme or DNA was added, and, as shown, resumption of deoxynucleotide incorporation recurred only after DNA addition. Thus, this DNA preparation had a limited ability to accept nucleotides; once these nucleotides were added, the DNA was no longer active as primer.

The ability of RLV polymerase to fill larger regions of sonically treated DNA subjected to exonuclease III attack was studied (Table 11). In the presence of Mg^{2+} as the only divalent

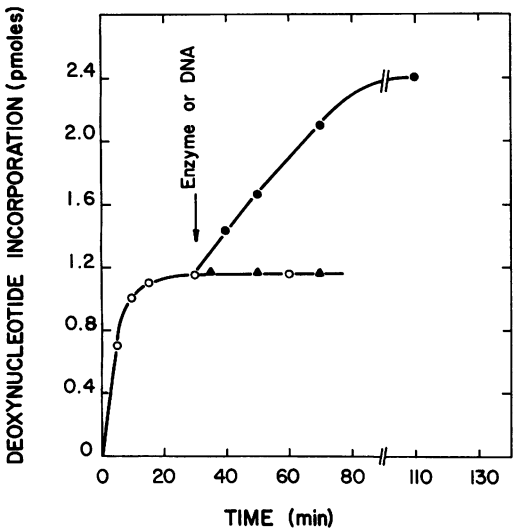

FIG. 3. *Extent of deoxynucleotide incorporation with exonuclease III-treated DNA. Reaction mixtures (0.05 ml) contained 5 nmoles each of dATP, dGTP, and dTTP, 1 nmole of α-^{32}P-dCTP (2,100 counts per min per pmole), 5 μg of albumin, 2.5 μmoles of Tris-hydrochloride (pH 8.0), 0.5 μmole of $MgCl_2$, 0.25 μmole of dithiothreitol, 0.25 μmole of KCl, 216 pmoles of sonically treated λ DNA treated with exonuclease III which had removed 2% of the total nucleotides, and 0.39 unit of Rauscher DNA polymerase (phosphocellulose eluate). Reactions were incubated for the indicated length of time; after 30 min, additional enzyme or DNA (216 pmoles) was added. The incorporation refers only to labeled nucleotide and should be multiplied by 4 to calculate total nucleotide incorporation. Symbols: (○) deoxynucleotide incorporation; (●) deoxynucleotide incorporation after addition of DNA; (▲) after addition of enzyme.*

TABLE 11. *Repair of sonically treated λ DNA treated with exonuclease III*[a]

DNA rendered acid-soluble by exonuclease III (%)	Yield of nucleotide incorporation (%)
0.21	0.20
0.86	0.88
2.04	2.02
4.0	4.2
10	5.3
	10.3 (+ Mn^{2+})
30	2.3
	21.3 (+ Mn^{2+})

[a] Reaction mixtures were as described in Fig. 3 with α-^{32}P-deoxyadenosine triphosphate (660 counts per min per pmole). The amount of DNA added was different in each case, e.g., (1) 3.81 nmoles, (2) 0.47 nmole, (3) 0.226 nmole, (4) 0.08 nmole, (5) 0.1 nmole, (6) 0.181 nmole. In reaction mixtures designated + Mn^{2+}, 0.05 μmole of $MnCl_2$ replaced $MgCl_2$. Incubation in all cases was for 60 min at 37 C. In experiments with extensively digested DNA (no. 5 and 6), the addition of more enzyme (0.39 unit) or extended incubation periods (up to 180 min) did not change the extent of nucleotide incorporation.

cation, limiting amounts of substrate, and large excess of polymerase, the extent of repair exactly matched the amount of nucleotides removed by exonuclease III up to about 100 nucleotides. When 250 nucleotides had been removed, the repair was only 50% of that expected; with 750 nucleotides removed, the activity was greatly reduced. As noted by Scolnick et al. (34), the activity of a template such as poly (A)·poly (U) is greatly increased in the presence of Mn^{2+}. A similar observation was noted in the repair of extensively single-stranded DNA.

Presence of various activities in RLV and AMV virions. Extracts of Rauscher and avian virions contain DNA-endonuclease activity. This activity was investigated in RLV by use of a highly specific assay for endonuclease activity producing 5′-PO_4 and 3′-OH termini. This assay takes advantage of the cleavage of the DNA ligase-AMP covalent linkage during the sealing of single-stranded breaks in DNA. In these experiments, *E. coli* DNA ligase was linked to ^{3}H-AMP (740 counts per min per pmole) after reaction with ^{3}H-AMP-labeled DPN. The product was isolated as previously described (32) and used to detect the introduction of single-strand breaks in intact λ DNA. In the presence of nonionic detergents such as Nonidet P-40 (NP-40) or Triton X-100, 10 nmoles of single-strand breaks were detected in 30 min per mg of protein; in the absence of NP-40 only 1 nmole per mg of protein per 30

min was detected. Mizutani et al. (23) have reported the presence of endonuclease, DNA ligase, and exonuclease in extracts of Rous sarcoma virus. Quintrell et al. (28), on the other hand, could not detect endonuclease activity in extracts of virus. At present, it is difficult to evaluate whether any activity is enclosed in the virion or associated with lipid-protein complexes which fortuitously band at the same density as virions. We have observed the presence of DNA ligase activity in extracts of virions as have Mizutani et al. (24). DNA ligase was measured by the circularization of ^{3}H-d(AT) (25). In some experiments, this activity has been stimulated by ATP, but the enzyme activity appears to be extremely labile and is readily removed from the DNA polymerase activity during purification. The final polymerase preparations described here are devoid of detectable ligase activity.

In addition, ribonuclease activity has been detected by Quintrell et al. (28) in Rous virions. We have found these enzymes in both AMV and RLV virions. At present, the properties of these nucleases are unknown.

DISCUSSION

The properties of the DNA-dependent deoxynucleotide incorporation catalyzed by both the RLV and AMV polymerases are similar to other DNA polymerases studied. The viral enzymes resemble DNA polymerase II and differ from DNA polymerase I in that they lack the two exonucleases (as measured with native DNA) which act in conjunction with polymerzing activity.

The results presented above indicate that the viral polymerases catalyze repair-like reactions dependent on a primer strand containing 3′-OH ends attached to a template strand which can be used to direct deoxynucleotide incorporation. It is clear that these enzymes, like *E. coli* DNA polymerases I and II and animal cell DNA polymerases, do not initiate new chains. [The exception to this generalization is the unprimed synthesis of poly d(AT) copolymer and poly (dI)·poly (dC) observed with DNA polymerase I (30, 33).]

A variety of synthetic polydeoxynucleotides as well as RNA-DNA hybrids will support deoxynucleotide incorporation. Baltimore and Smoler (5) observed a preference for the incorporation of deoxynucleotides onto polydeoxynucleotide chains rather than onto polyribonucleotide components of RNA-DNA hybrids. At present, it is not clear whether this preference reflects the specificity of these polymerases or is due to the complexity of polynucleotide structures induced by the presence of 2′-OH groups in polyribonucleotides. It is clear that poly (A)·poly (dT) and poly (I)·poly (dC) preferentially support dTMP and dCMP incorporation, respectively, in agreement with the observations of Baltimore and Smoler (5) as well as Spiegelman et al. (36).

The efficiency of priming by different synthetic polynucleotides depends upon the extent of single-stranded regions which can be repaired. Thus, the utilization of synthetic polynucleotides as a means of detecting and differentiating polymerases can be misleading. This is particularly true if nucleases contaminate polymerase preparations.

The heteropolymeric duplexes which best support deoxynucleotide incorporation are those rich in A and T. This observation is not surprising considering the multiple structures that these polymers can assume (25). At the temperatures used to study most polymerization reactions (30 to 37 C), AT-rich polymers can melt, show "creeping" phenomena, and form multiple structures which are in equilibrium with one another. Thus, polymerases [or ligases (25)] can displace this equilibrium by the preferential utilization of a duplex structure. Contrasted to this, GC-rich polymers, with their high T_m, are more rigid and thus less efficient unless small oligomeric structures are used, as described by Baltimore and Smoler (5). It is not surprising that there are marked differences in results between laboratories when GC-rich polymers are compared. The efficiency of priming with such polymers will depend upon the primer-template relationship described above; this relationship will depend upon the structure of each of the homopolymers and conditions used for interactions.

A further striking similarity between DNA polymerase II of *E. coli* and the RNA tumor virus polymerases is the inability of these enzymes to repair extensive single-stranded regions of DNA. The substrates used here (Table 11) are those which were used with DNA polymerase II (41). Identical results were obtained regarding the lack of repair of DNA containing extensive single-stranded regions. Similar findings with DNA polymerase II were made by Kornberg and Gefter (14), who in collaboration with Alberts and co-workers have shown that *E. coli* contains a protein factor (resembling the T4 gene 32 protein) which permits DNA polymerase II to repair extensive single-stranded DNA (M. Gefter, *personal communication*). This protein factor is specific for *E. coli* DNA polymerase II and does not influence T4 DNA polymerase. Similarly, the T4 gene 32 protein discovered by Alberts et al. (1) only permits the T4 DNA polymerase to repair stretches of single-stranded regions and does not affect DNA polymerase II. A similar protein may be present in the virion which would allow

more extensive synthesis than currently observed with viral RNA templates.

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