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Studies on Reverse Transcriptase of RNA Tumor Viruses
III. Properties of Purified Moloney Murine Leukemia Virus DNA Polymerase and Associated RNase H

INDER M. VERMA

Tumor Virology Laboratory, The Salk Institute, San Diego, Calfornia 92112

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DNA polymerase was purified from a cloned isolate of Moloney murine leukemia virus (M-MuLV). Purified M-MuLV DNA polymerase, upon analysis by polyacrylamide gel electrophoresis, showed one major polypeptide of mol wt 80,000. Estimation of molecular weight from the sedimentation rate of the purified enzyme in a glycerol gradient was consistent with a structure containing one polypeptide. M-MuLV DNA polymerase could transcribe ribopolymers, deoxyribopolymers, and heteropolymers as efficiently as did purified DNA polymerase from avian myeloblastosis virus (AMV). M-MuLV DNA polymerase, however, transcribed native 70*S* viral RNA less efficiently than did AMV DNA polymerase. Addition of oligo(dT) enhanced five to tenfold the transcription of 70*S* viral RNA by M-MuLV DNA polymerase. Purified enzyme also exhibited nuclease activity (RNase H) that selectively degraded the RNA moiety of the RNA-DNA hybrid. It did not degrade single-stranded RNA, single-stranded DNA, double-stranded RNA, and double-stranded DNA. M-MuLV DNA polymerase-associated RNase H acted as a random exonuclease. When [^{3}H]poly(A)·poly(dT) was used as a substrate, the size of the M-MuLV DNA polymerase-associated RNase H digested product was larger than the size of the digestion products by AMV DNA polymerase. The oligonucleotide digestion products could be further digested to 5′-AMP by snake venom phosphodiesterase, indicating that the products were terminated by 3′-OH groups. Alkaline hydrolysis of the oligonucleotide digestion products generated pAp, suggesting that M-MuLV DNA polymerase-associated RNase H cleaves at the 3′ side of the 3′,5′-phosphodiester bond. The ratios of the rates of DNA polymerase activity and RNase H activity were not significantly different in the murine and avian enzymes.

RNA tumor viruses contain a DNA polymerase that can utilize both polyribonucleotides and polydeoxyribonucleotides as templates to synthesize complementary DNA (1, 3, 28, 30, 31). Purified DNA polymerase from avian RNA tumor viruses also exhibits a nuclease activity that can selectively degrade the RNA moiety of an RNA-DNA hybrid (4, 18, 21, 25). The nuclease activity has been referred to as RNase H (25). DNA polymerase purified from avian DNA tumor viruses contains two subunits, α (mol wt of 62,000) and β (mol wt of 95,000) (11, 13, 17, 30, 35, 36, 39). The isolated α subunit can manifest both the DNA polymerase and RNase H activities (13). Thermal inactivation studies performed on the purified DNA polymerases from two temperature-sensitive mutants of Rous sarcoma virus (RSV) suggest that the α subunit manifesting the polymerase and nuclease activities is coded for by the viral RNA (34, 36). The β subunit has been shown to enhance the affinity of α to the template or substrate (14; A. Panet, I. M. Verma, and D. Baltimore, Cold Spring Harbor Symp. Quant. Biol., in press). The suggestion that α may be a proteolytic cleavage product of β (K. Mölling, Cold Spring Harbor Symp. Quant. Biol., in press) and that there are similarities between α and β (11) strongly indicates that the β subunit may be the precursor of the α subunit.

DNA polymerase from murine leukemia viruses (MuLV) has been shown to be a single polypeptide with a mol wt of 70,000 to 80,000 (9, 15, 32, 39, 41; K. Mölling, Cold Spring Harbor Symp. Quant. Biol., in press). MuLV DNA polymerase does not transcribe 70*S* viral genome RNA as efficiently as DNA polymerase from avian RNA tumor viruses (41). Some reports indicate that RNase H activity can be dectected in purified MuLV DNA polymerase

(12, 42; Mölling, in press); other reports indicate that no RNase H activity can be detected (22, 26, 39, 41, 44). Recently, Wu et al. (44) reported that the RNase H and DNA polymerase activities from purified preparations of Rausher MuLV DNA polymerase can be separated by column chromatography. Furthermore, RNase H activity could not be detected in purified core structures, and a specific antibody to Raushcer MuLV DNA polymerase did not inhibit virion-associated RNase H activity.

Since purified DNA polymerase manifesting RNase H activity from RSV is coded for by the viral RNA (34, 36), it was of interest to study the presence of RNase H activity in purified MuLV DNA polymerase. We studied the properties of purified DNA polymerase from cloned isolates of Moloney MuLV (M-MuLV). Purified M-MuLV DNA polymerase has one polypeptide chain with an average mol wt of 80,000 and can transcribe ribohomopolymers, deoxyribohomopolymers, heteropolymers, 70*S* viral RNA, and natural RNAs. The purified enzyme exhibits RNase H activity that acts as a random exonuclease. The digestion products of the RNase H activity have a 5'-phosphate and a 3'-OH end. The RNase H digested products are 4 to 30 nucleotides long. The properties of M-MuLV DNA polymerase are compared with purified DNA polymerase from avian myeloblastosis virus (AMV).

MATERIALS AND METHODS

Materials. M-MuLV, clone 1, was generously supplied by P. MacIsaac and H. Fan of the Salk Institute. The details of the isolation of M-MuLV clone 1 have been described elsewhere (6). AMV was provided by the Office of Program Resources and Logistics of the Virus Cancer Program of the National Cancer Institute. Nonidet P-40 was purchased from Particle Data Laboratory, Elmhurst, Ill.; tritium- and ^{32}P-labeled deoxyribonucleoside triphosphates, unlabeled deoxyribonucleoside triphosphate, tritium-labeled ribonucleoside triphosphates, and 5'-ADP were obtained from New England Nuclear Corp., Schwarz/Mann Research, and P-L Biochemicals. [α-^{32}P]-TTP was obtained from ICN, Irvine, Calif. Tritium-labeled and unlabeled polymers were purchased from Miles Laboratories, P-L Biochemicals, and Collaborative Research, Inc. Oligonucleotides were 8 to 18 nucleotides long and were obtained from Collaborative Research, Inc. *Escherichia coli* RNA polymerase (Sigma Chemicals) was a gift of L. T. Bacheler and M. Vogt of the Salk Institute. ϕX174 DNA was a gift of M. Hayashi, University of California, San Diego. Polynucleotide phosphorylase (*Micrococcus lysodeikticus*) was obtained from Miles Laboratories. Covalently closed circular DNA of the colicinogenic factor E1 (Col E1) of *E. coli* was a gift of R. Leavitt and V. Hirshfield of the University of California, San Diego. *E. coli* RNase H was a gift from both M. J. Rosenfeld of the University of California, San Diego, and Yoram Groner, Albert Einstein College of Medicine, Bronx, N.Y. Oligonucleotides $(A)_9A$, $(A)_6A$, $(A)_4A$, and $(A)_2A$ were purchased from Boehringer Mannheim Corp. Some A-oligomers were gifts of M. G. Sarngadharn, National Cancer Institute, Bethesda, Md. 5'-AMP and 3'-AMP were purchased from Sigma Chemicals. Snake venom phosphodiesterase was purchased from Calbiochem. 10*S* rabbit retriculocyte RNA was a gift of G. F. Temple of the Massachusetts Institute of Technology, Cambridge, and 18*S* slime mold RNA was a gift of R. A. Firtel of the University of California, San Diego.

Purification of DNA polymerase. Sixty milligrams of sucrose gradient-purified M-MuLV was lysed with Nonidet P-40 (final concentration of 0.05%) and the lysate was incubated at 37 C for 2 min. DNA polymerase was purified from the lysate by chromatography on a DEAE-Sephadex (A-25) column, followed by chromatography on a phosphocellulose column as described (35). The peak of enzyme activity from the DEAE-Sephadex column eluted at a salt concentration of 0.07 M KCl and from the phosphocellulose column at a salt concentration of 0.24 M KCl (Fig. 1A). Enzyme from fractions 49 to 53 of the phosphocellulose column (Fig. 1A) was used in the experiments reported here.

AMV DNA polymerase was purified as described (35). Rauscher MuLV DNA polymerase was purified in collaboration with D. Smoler of the Massachusetts Institute of Technology as described (35).

Polymerase assay. The standard reaction mixture for DNA polymerase activity in 0.1 ml contained 50 mM Tris-hydrochloride (pH 8.3), 10 mM dithiothreitol (DTT), 1 mM Mn^{2+}, 60 mM NaCl, 10 nmol of [^{3}H]dTTP (50 counts/min per mol), 2.8 nmol of poly(A), and 0.84 nmol of oligo(dT). When poly(dC) or poly(C) was used as template, 10 mM Mg^{2+}, 10 nmol of [^{3}H]dGTP (50 counts/min per pmol), and 0.84 nmol of oligo(dG) replaced Mn^{2+}, [^{3}H]dTTP, and oligo(dT), respectively. The reaction mixture was incubated at 37 C for given periods of time, and acid-precipitable radioactivity was determined as described (2). All enzyme reactions were carried out for times during which synthesis was a linear function of time.

Unit of activity. A unit of enzyme activity is defined as the amount of enzyme required to incorporate 100 pmol of TMP at 37 C in 15 min in a standard polymerase assay mixture. Equal units of enzyme activity were used when comparisons of M-MuLV DNA polymerase and AMV DNA polymerase were made.

Glycerol gradients. Twenty-nine units of M-MuLV DNA polymerase contained in 0.03 ml was diluted with 0.07 ml of 0.01 M Tris-hydrochloride (pH 7.5) and layered on a 20 to 40% glycerol gradient containing 50 mM Tris-hydrochloride (pH 8.3), 100 mM β-mercaptoethanol, 1 mM EDTA, 300 mM KCl, and 0.2% Nonidet P-40. The gradient was centrifuged for 27 h at 45,000 rpm at 4 C in an SW 50.1 rotor of a Spinco ultracentrifuge. Fractions (0.1 ml) were collected by puncturing the bottom of the centrifuge

tube, and aliquots from every other fraction were withdrawn for polymerase and RNase H assays. *E. coli* DNA polymerase I (0.24 μg) was included as a standard. In a parallel gradient, 22 U of AMV DNA polymerase and 0.24 μg of *E.coli* DNA polymerase I were centrifuged. AMV DNA polymerase and *E. coli* DNA polymerase I were assayed as described (39). Over 75% of the input enzyme activity of M-MuLV DNA polymerse, AMV DNA polymerase, and *E. coli* DNA polymerase I was recovered from the glycerol gradient.

Polyacrylamide gel electrophoresis. A 200-μl sample from fraction 52 of a phosphocellulose column (180 U of activity) was precipitated with 10% trichloroacetic acid and analyzed on polyacrylamide-methylene bisacrylamide slab gels (10), using a discontinuous buffer system containing sodium dodecyl sulfate (19). The protein bands were located in the gel by staining with Coomassie brilliant blue as described (10,11). The standards included AMV DNA polymerase, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c.

Synthesis of the φX174 DNA-[^{3}H]RNA hybrid. The procedure described by Stravrianopoulos et al. (29) to synthesize the f_1 DNA-RNA hybrid was followed with some modifications. Briefly, the reaction mixture contained, in 0.5 ml, 80 mM Tris-hydrochloride (pH 8.9), 50 mM KCl, 10 mM DTT, 10 mM Mn^{2+}, 60 μg of φX174 (N-11 E mutant; isolated in M. Hayashi's laboratory) DNA, 10 mM each ATP, CTP, and GTP, 4.2 nmol of [^{3}H]UTP (1.33×10^4 counts/min per pmol), and 20 μl of *E. coli* RNA polymerase (395 U/mg). After 5 h of incubation at 37 C, the hybrid was separated from unincorporated radioactive material by chromatography on a Sephadex G-50 column (34). The peak fractions of radioactivity eluting in the void volume were collected, lyophilized, and dissolved in 0.3 ml of buffer containing 10 mM Tris-hydrochloride (pH 7.5) and 10 mM NaCl; 0.03 ml of buffer containing 0.5 M NaCl, 0.01 M EDTA, and 0.1 M *N*-Tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (pH 7.0) was added to the solution containing the hybrid, and the mixture was transferred to a tightly sealed tube and immersed in a water bath at 68 C for 60 min. The contents of the tube were chilled in ice-cold water. The hybrid was over 95% resistant to digestion by RNase reagent (400 μg of pancreatic RNase A per ml + 80 μg of RNase T1 per ml contained in 0.01 M Tris-hydrochloride [pH 7.5] and 0.01 M NaCl) in 0.3 M NaCl and 0.03 M sodium citrate.

Synthesis of [^{3}H]poly(A). [^{3}H]poly(A) was synthesized as described (4). Briefly, the reaction mixture in 1.0 ml contained 100 mM glycine (pH 9.0), 10 mM Mg^{2+}, 0.4 mM EDTA, 100 μg of bovine serum albumin, 50 nmol of [^{3}H]ADP (1,000 counts/min per pmol), and 4.8 U of polynucleotide phosphorylase. After 60 min at 37 C, over 10% of the radioactivity was acid insoluble, and the product was twice extracted with phenol followed by two extractions with chloroform-isoamyl alcohol. The product was purified on a Sephadex G-50 column (as described above), and peak fractions were pooled, lyophilized, and dissolved in a buffer containing 10 mM Tris-hydrochloride (pH 7.5) and 10 mM NaCl. The average chain length of [^{3}H]poly(A) synthesized was 300 nucleotides. Some experiments were performed with [^{3}H]poly(A) provided by S. Drost of the Massachusetts Insitute of Technology.

Synthesis of [^{32}P]poly(dT). [^{32}P]poly(dT) was synthesized by using poly(A)·oligo(dT) as template primer and AMV DNA polymerase. The same reaction conditions as described for the DNA polymerase assay were used except that 10 nmol of α[^{32}P]dTTP (1,000 counts/min per pmol) was added. The reaction was stopped when over 10% of the radioactivity had been rendered acid insoluble. The template was hydrolyzed by boiling in 0.3 N NaOH for 5 min. The product was purified on a Sephadex G-50 column (as described above), and peak fractions were pooled, lyophilized, and dissolved in water.

RNase H assay. The standard reaction mixture for RNase H assays contained, in 0.1 ml, 50 mM Tris-hydrochloride (pH 8.3), 10 mM DTT, 1 mM Mn^{2+}, 30 mM NaCl, and 12 pmol of the φX174 DNA-[^{3}H]RNA hybrid or [^{3}H]poly(A)·poly(dT) (ratio 1:0.3). Incubations were carried out at 37 C, and either the residual acid-insoluble radioactivity or acid-soluble radioactivity (36) was determined by adding to the reaction mixture 40 μg of bovine serum albumin and 4 μl of 100% trichloroacetic acid. The reaction mixture was centrifuged in a bench-top centrifuge, and the supernatant was counted directly in 5.0 ml of scintillation fluid (Aquasol).

Sucrose gradients. φX174 DNA-[^{3}H]RNA hybrids were analyzed on 5 to 20% sucrose gradients containing 100 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), and 1 mM EDTA. The gradients were centrifuged at 45,000 rpm for 3.5 h at 4 C in an SW50.1 rotor of a Spinco ultracentrifuge. Five-drop fractions were collected by puncturing the bottom of the centrifuge tube, and radioactivity was counted in 5.0 ml of Aquasol.

The Col E1 plasmid DNA was analyzed by centrifugation on 5 to 20% sucrose gradients containing 50 mM Tris-hydrochloride (pH 7.5), 500 mM NaCl, and 5 mM EDTA for 2.5 h at 45,000 rpm at 4 C in an SW50.1 rotor. Fractions were collected and radioactivity was determined as described above.

Chromatography and high-voltage paper electrophoresis. The digestion products of [^{3}H]poly(A)·poly(dT) were spotted on a 56-cm strip of Whatman no. 1 paper and chromatographed in *n*-propanol-ammonia-water (55:10:35) to separate various oligomers of AMP (20). The paper was air dried, 1-cm strips were cut, and radioactivity was determined in toluene-based scintillation fluid (80 ml of Liquifluor in 2.54 liters of toluene).

High-voltage paper electrophoresis was performed on alkali-digested acid-soluble digestion products in buffer containing 0.03 M KH_2PO_4 (pH 7.1 to 7.4). Electrophoresis was carried out for 45 min at 3 kV. The paper was air dried and radioactivity was counted as described above.

RESULTS

Properties of purified M-MuLV DNA polymerase. The chromatographic behavior of

M-MuLV DNA polymerase on a phosphocellulose column is shown in Fig. 1A. The RNA-directed DNA polymerase activity measured by poly(A)·oligo(dT)-directed poly(dT) synthesis, DNA-directed DNA polymerase activity measured by poly(dC)·oligo(dG)-directed poly(dG) synthesis, and RNase H activity measured by degradation of the RNA moiety of the ϕX174 DNA-[^{3}H]RNA hybrid all co-chromatographed on the phosphocellulose column. Further purification of the DNA polymerase and RNase H activities by sedimentation through a glycerol gradient showed that the two activities sedimented together (Fig. 1B). The purity of the enzyme was analyzed by polyacrylamide gel electrophoresis. M-MuLV DNA polymerase purified by phosphocellulose chromatography showed one major polypeptide band with an average mol wt of 80,000 and a faint band with an average mol wt of 15,000 (Fig. 2, panel II). Polyacrylamide gel analysis of the enzyme after further purification by velocity sedimentation in a glycerol gradient indicated that the low-molecular-weight component had been separated from the 80,000-dalton component (data not shown). The molecular weight of M-MuLV DNA polymerase was different from that of either the α or β subunit of AMV DNA polymerase (Fig. 2, panel I).

The molecular weight of M-MuLV DNA polymerase was also estimated by sedimentation on glycerol gradients (Fig. 1B). Based upon the sedimentation values of AMV DNA polymerase (7.5S) and that of *E. coli* DNA polymerase I (5.6S) (16), M-MuLV DNA polymerase had an apparent sedimentation coefficient of 4.5S. By

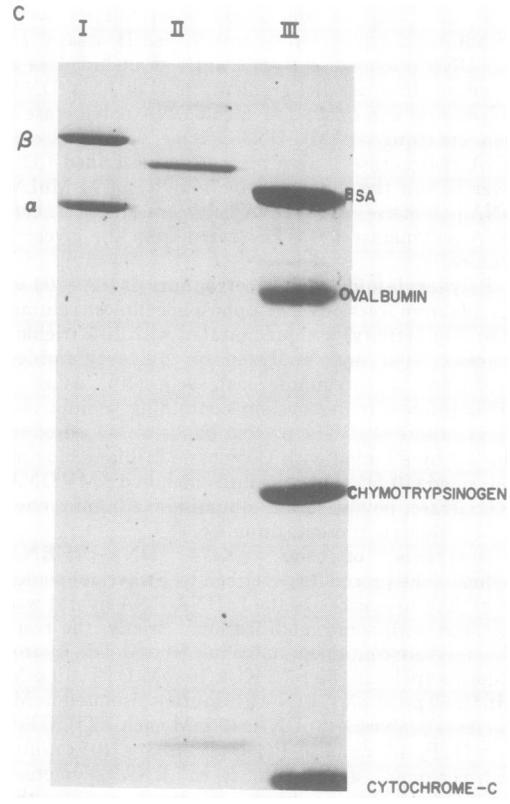

FIG. 2. *Polyacrylamide gel electrophoresis. (I) AMV DNA polymerase; the mol wt of β and α subunits are 98,000 and 62,000, respectively. (II) M-MuLV DNA polymerase. (III) Standard proteins. The mol wt of the standards were: bovine serum albumin (BSA), 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; cytochrome c, 11,700.*

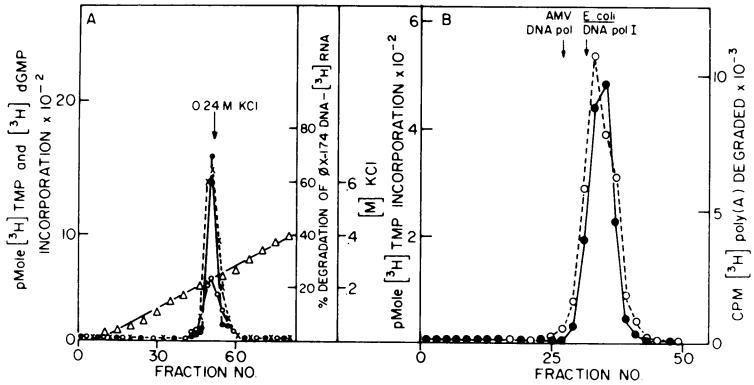

FIG. 1. *Purification and molecular weight determination of M-MuLV DNA polymerase. (A) Elution profile of poly(A)·oligo(dT)-directed poly(dT) synthesis, poly(dC)·oligo(dG)-directed poly(dG) synthesis, and RNase H activity from the phosphocellulose column. Symbols: (●) [^{3}H]TMP incorporation (4 μl of every other fraction was assayed); (○) [^{3}H]dGMP incorporation (2 μl of every other fraction was assayed); (×) RNase H activity (5-μl fractions were assayed); (Δ) salt gradient. (B) Glycerol gradients. The figure is drawn by aligning the peak of enzymatic activity of E. coli DNA polymerase I in two separate gradients containing either M-MuLV DNA polymerase or AMV DNA polymerase. The arrows indicate the sedimentation position of AMV DNA polymerase and E. coli DNA polymerase. Symbols: (●) [^{3}H]TMP incorporation; (○) RNase H activity.*

using the formula (mol $wt_1)^{2/3}$/mol $wt_2 = S_1/S_2$ (24), the molecular weight of M-MuLV DNA polymerase has been calculated to be 80,000. This is in close agreement with the molecular weight values obtained here by polyacrylamide gel electrophoresis and with the report that the DNA polymerase purified from the Friend MuLV complex has a mol wt of 84,000 (Mölling, in press).

A comparison of transcription of various polyribonucleotides and polydeoxyribonucleotides by M-MuLV DNA polymerase and AMV DNA polymerase is shown in Table 1. Most templates tested were transcribed efficiently by the two enzymes. However, 70*S* RNA from either M-MuLV or AMV was five to ten times less efficiently transcribed by M-MuLV DNA polymerase than AMV DNA polymerase. Oligo(dT) was added to the reaction mixture to test whether the exogenous primer could enhance the transcription of 70*S* viral RNA by M-MuLV DNA polymerase. The synthesis of DNA complementary to 70*S* RNA by M-MuLV DNA polymerase was stimulated 10-fold by the exogenous primer. 10*S* rabbit reticulocyte RNA in the presence of oligo(dT) and actinomycin D (100 μg/ml) and 18*S* slime mold rRNA in the presence of oligo(dC) could be transcribed into complementary DNA by both enzymes. When 10*S* rabbit reticulocyte RNA·oligo(dT) is used as template-primer in the absence of actinomycin D, complementary double-stranded DNA is synthesized (35). Thus it appears that the two enzymes can transcribe a variety of templates with equal efficiency except for native viral 70*S* RNA without exogenous primer.

The choice of divalent cations and their concentration can markedly affect the transcription of certain templates by M-MuLV DNA polymerase (Table 2). At optimum Mn^{2+} concentration (1 mM), poly(A)·oligo(dT)-directed poly(dT) synthesis was three times more efficient than at the optimum Mg^{2+} concentration (2 mM). However, poly(C)·oligo(dG)-directed or poly(dC)·oligo(dG)-directed poly(dG) synthesis was four to eight times more efficient at the optimum Mg^{2+} concentration (10 mM) than at the optimum Mn^{2+} concentration (1 to 2 mM). The RNase H activity was also more efficient with Mn^{2+} than Mg^{2+} (Table 2).

The RNase H activity associated with either AMV DNA polymerase or RSV DNA polymerase has been shown to be more resistant to thermal inactivation than the polymerase activity (22, 36): Similarly, the DNA polymerase activity of M-MuLV DNA polymerase is threefold more labile than RNase H activity (Table 3). Unlike AMV or RSV DNA polymerase (38; Panet et al., in press), the addition of template primer during preincubation did not protect the M-MuLV DNA polymerase from heat inactivation. In this respect, the thermal inactivation properties of M-MuLV DNA polymerase are similar to the inactivation properties of the α subunit of AMV (Panet et al., in press).

TABLE 1. *Transcription of ribopolymers and deoxyribopolymers*[a]

Template-primer	^{3}H-labeled substrate	M-MuLV DNA polymerase (pmol)	AMV DNA polymerase (pmol)
Poly(A)·oligo(dT)	dTTP	7,280	7,104
Poly(C)·oligo(dG)	dGTP	4,362	7,000
70*S* AMV RNA with Mg^{2+}	dATP	3.5	15.5
70*S* AMV RNA with Mn^{2+}	dATP	1.25	
70*S* AMV RNA with Mg^{2+} + oligo(dT)	dATP	36	60
70*S* M-MuLV clone 1 RNA with Mg^{2+}	dATP	6.6	50
70*S* M-MuLV clone 1 RNA with Mn^{2+}	dATP	1.45	
10*S* rabbit reticulocyte globin RNA + oligo(dT)	dGTP	500	595
10*S* rabbit reticulocyte globin RNA + oligo(dT) + actinomycin D	dGTP	300	406
18*S* slime mold rRNA + oligo(dC)	dATP	14	16
Poly(dC)·oligo(dG)	dGTP	11,890	4,500
Poly(dA-dT)	dTTP	234	254
Activated DNA	dTTP	45	39
Native calf thymus DNA	dTTP	13	5.5
Heat-denatured calf thymus DNA	dTTP	11	8.0

[a] The standard polymerase reaction mixture was used with appropriate template-primer, substrate, and divalent cations. 14 nmol of poly(A), poly(C), and poly(dC) and 3.5 nmol of oligo(dT) and oligo(dG) were used. 1.4 nmol of 70*S* AMV RNA, 2.5 nmol of 70*S* M-MuLV RNA, 5.6 nmol of 10*S* rabbit reticulocyte RNA, 1.4 nmol of ^{32}P-labeled 18*S* rRNA from slime mold (2×10^6 counts/min per μg), and 1.5 nmol of oligo(dT) and oligo(dC) as primers were used. 5.6 nmol each of poly(dA-dT) copolymer, activated DNA (gift of Tom Harrison and R. P. McCaffrey, Massachusetts Institute of Technology), native and heat denatured calf thymus DNA were used. 10 nmol of radioactive deoxyribonucleoside triphosphate (100 counts/min per pmol) and 1 mM each unlabeled deoxyribonucleoside triphosphates were used. 1 mM Mn^{2+} was used in the reaction mixture containing 70*S* RNA (where indicated). Actinomycin D was used to a final concentration of 100 μg/ml. 18.2 U of M-MuLV DNA polymerase and 17.7 U of AMV DNA polymerase were added in each reaction mixture, and the incubations were carried out for 60 min at 37 C. The table summarizes a comparison of the rates of reaction by the two enzymes.

RNase H activity. The RNase H activity of purified M-MuLV DNA polymerase was assayed by using the φX174 DNA-[^{3}H]RNA hybrid as substrate. Ninety percent of the RNA of the φX174 DNA-[^{3}H]RNA hybrid was rendered acid soluble in 30 min by M-MuLV DNA

TABLE 2. *Effect of divalent ions on DNA polymerase and RNase H activities*[a]

Enzyme	Mg^{2+}			Mn^{2+}		
	Optimum mM	Incorporation (pmol)	% degraded	Optimum mM	Incorporation (pmol)	% degraded
DNA polymerase						
Poly(A)·oligo(dT)	2	74		1	260	
Poly(C)·oligo(dG)	10	219		1	30	
Poly(dC)·oligo(dG)	10	420		2	100	
RNase H						
φX174 DNA-[^{3}H]RNA	1-2		>70	0.1-1.0		>90
[^{3}H] poly(A)·poly(dT)	2		20	2		50

[a] The standard polymerase reaction mixture contained 2.8 nmol of template, 0.8 nmol of primer, and 2.25 U of M-MuLV DNA polymerase. Incubations were carried out for 15 min. For RNase H assays, 12 pmol of hybrid, or 21 pmol of [^{3}H]poly(A) and 13 pmol of poly(dT), was used as substrate and 4 U of enzyme was added. Incubations were carried out for 45 min.

TABLE 3. *Average half-time ($t_{1/2}$) of DNA polymerase and RNase H activities*[a]

Source of DNA polymerase	$t_{1/2}$ in min at 45 C		
	DNA polymerase activity		RNase H activity
	Preincubation without template-primer	Preincubation with template-primer	
M-MuLV	6.5	6.5	18
Rauscher MuLV	6.0	6.5	
AMV	12		15

[a] Heat inactivations were performed as described (36). Briefly, 0.3 ml of reaction mixture containing 100 mM Tris-hydrochloride (pH 8.3), 20 mM DTT, 2 mM Mn^{2+}, 120 mM NaCl, and 10 U of enzyme was incubated at 45 C. At various time intervals, 0.05 ml of the reaction mixture was withdrawn, added to 0.05 ml of reaction mixture II containing 2.8 nmol of poly(A), 0.8 nmol of oligo(dT), and 10 nmol of dTTP (100 counts/min per pmol), and incubated for 15 min at 37 C. Acid-precipitable radioactivity was determined as described (2). Where the preincubation mixture contained template-primer, it was omitted from reaction mixture II. For RNase H assay, the substrate was omitted from the preincubation reaction mixture. The blanks indicate that tests were not performed on these samples.

polymerase (Fig. 3A). If the enzyme was omitted from the reaction mixture or the hybrid was boiled for 2 min before being added to the reaction mixture, less than 5% of the RNA of the hybrid was rendered acid soluble. Thus it appears that the RNA moiety of this hybrid is susceptible to RNase H activity only in RNA-DNA hybrid form. The degradation of the hybrid was also analyzed by sedimentation on sucrose gradients (Fig. 3B). Over 63 and 85% of the RNA was degraded after incubation for 15 and 30 min, respectively. The degradation of the RNA moiety of the hybrid was accompanied by a decrease in the amount of radioactivity in the 23*S* region of the sucrose gradients and a corresponding increase in the amount of radioactivity at the top of the gradient. This suggests that the product of digestion is not hydrogen bonded to DNA template.

Table 4 shows the properties of M-MuLV DNA polymerase-associated RNase H activity analyzed by using several substrates. Over 50% of [^{3}H]poly(A) and 64% of [^{3}H]poly(C) were degraded when complementary poly(dT) and ply(dG) were synthesized, whereas in the absence of DNA synthesis (without primer) no degradation of [^{3}H]poly(A) or [^{3}H]poly(C) was observed. The synthesis of complementary DNA was, however, not obligatory for RNase H activity since preformed hybrids, [^{3}H]poly(A)·poly(dT), [^{3}H]poly(C)·poly(dG), and [^{3}H]poly(U)·poly(dA) were susceptible to degradation by the enzyme. The efficiency of degradation of various hybrids depended on the nature of the substrate; e.g., poly(A) in [^{3}H]poly(A)·poly(dT) was degraded more efficiently than poly(U) in the [^{3}H]poly(U)·poly(dA) hybrid. Over 86% of the poly(A) in [^{3}H]poly(A)·[^{32}H]poly(dT) was degraded without loss of ^{32}P-containing acid-insoluble radioactivity. These results suggest that (i) only the RNA moiety of an RNA-DNA hybrid is susceptible to RNase H activity, (ii) the enzyme is free of DNase activity, and (iii) M-MuLV-associated RNase H is unable to digest double-stranded RNA, [^{3}H]poly(A)·poly(U), or double-stranded DNA, [^{32}P]poly(dT)·poly(dA).

In a previous report (41), it was shown that the ratio of RNase H activity to DNA polymer-

ase activity from Kirsten murine sarcoma-leukemia virus is very low. We determined the relative amounts of RNase H and DNA polymerase activities of M-MuLV DNA polymerase and AMV DNA polymerase (Table 5). These comparisons are based on the rate of synthesis of poly(dT) and rate of degradation of poly(A) and not on the net synthesis or degradation. The ratio of DNA polymerase to RNase H activity of the two enzymes was not significantly different.

Mode of action of RNase H. (i) Does the enzyme act as an endonuclease or an exonuclease? RNase H of cellular origin acts as an endonuclease (18), whereas RNase H associated with AMV DNA polymerase has been shown to act as an exonuclease (14, 18, 21). To determine whether RNase H associated with M-MuLV DNA polymerase acts as an endonuclease, we used a closed circular superhelical DNA containing ribonucleotides as substrate. When the plasmid Col E1 replicates in the

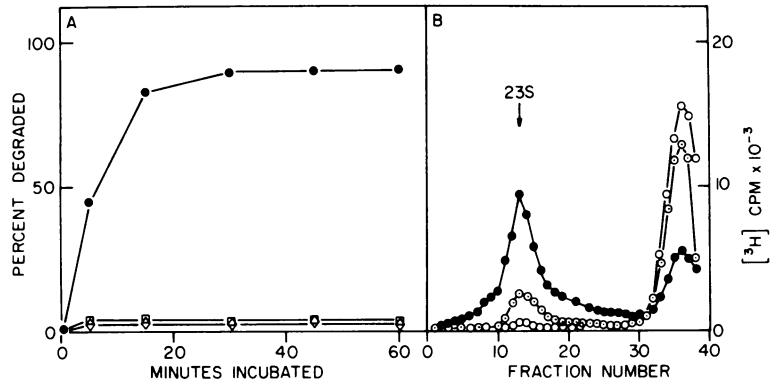

FIG. 3. *RNase H activity of M-MuLV DNA polymerase. (A) Kinetics of degradation of φX174 DNA-[³H]RNA hybrid. 12 pmol of hybrid and 40 U of M-MuLV DNA polymerase were added to a standard reaction mixture. At various times after incubation, 15-μl samples were withdrawn and acid-precipitable radioactivity was determined. Symbols: (●) Complete reaction mixture; (□) boiled hybrid; (◇) complete reaction mixture without enzyme. (B) Sedimentation profile of the hybrid after incubation with M-MuLV DNA polymerase. 12 pmol of hybrid and 13 U of enzyme were incubated in a standard reaction mixture for 15 and 30 min and centrifuged on sucrose gradients. Symbols: (●) Hybrid incubated for 30 min without enzyme; (⊙) hybrid incubated for 15 min with enzyme; (○) hybrid incubated for 30 min with enzyme.*

TABLE 4. *Properties of RNase H activity of purified M-MuLV DNA polymerase*[a]

Template-primer or substrate	Degradation (%)		Incorporation (pmol)	
	³H	³²P	[³²P]dTMP	[³H]dGMP
[³H]poly(A)·oligo(dT)	53		253	
[³H]poly(A)	<4		0	
[³H]poly(C)·oligo(dG)	64			1,433
[³H]poly(C)	1			0
[³H]poly(A)·[³²P]poly(dT)	86	0		
[³H]poly(U)·poly(dA)	12			
[³H]poly(A)·poly(U)	<3			
[³²P]poly(dT)·poly(dA)		<1		

[a] The DNA polymerase reaction mixture contained either 30 pmol of [³H]poly(A), 15 pmol of poly(dT), 2 nmol of [³²P]dTTP (400 counts/min per pmol), and 5 U of enzyme, or 2 nmol of [³H]poly(C) (3 counts/min per pmol), 0.7 nmol of oligo(dG), 2 mM [³H]dGTP (100 counts/min per pmol), and 25 U of enzyme. Incubations were carried out for 15 min. The same amount of template was added to the reaction mixture, where the primer was omitted. For RNase H assays, the substrate [³H]poly(A)·[³²P]poly(dT) contained 30 pmol of [³H]poly(A) and 20 pmol of [³²P]poly(dT); [³H]poly(U)·poly(dA) contained 20 pmol of [³H]poly(U) (300 counts/min per pmol) and 14 pmol of poly(dA). [³H]poly(A)·poly(U) contained 12 pmol of [³H]poly(A) and 11 pmol of poly(U); [³²P]poly(dT)·poly(dA) contained 26.6 pmol of [³²P]poly(dT) and 28 pmol of poly(dA). 5 U of M-MuLV DNA polymerase was added to each reaction mixture and incubated for 60 min. Acid-insoluble radioactivity was determined as described (2).

TABLE 5. *Relative rates of DNA polymerase and RNase H activities of M-MuLV DNA and AMV DNA polymerases* [a]

DNA polymerase	Poly(A) degraded (pmol)	Poly(dT) synthesized (pmol)	Ratio DNA polymerase/ RNase H
M-MuLV	56.7	610	10.7
AMV	67.3	592	8.9

[a] The standard DNA polymerase reaction mixture described in Materials and Methods was used for both enzymes. For RNase H assay, 110 pmol of [^{3}H]poly(A) (100 counts/min per pmol) and 42 pmol of poly(dT) were added to the reaction mixture. 18 U of M-MuLV DNA polymerase and AMV DNA polymerase enzyme was used for both the polymerase and RNase H assays, and incubations were carried out for 5 min at 37 C. The rates of reactions are compared. The data are an average of three experiments.

presence of chloramphenicol, some of its DNA contains ribonucleotides covalently inserted in one of the two DNA strands (5). The average chain length of covalently inserted ribonucleotides is 25 nucleotides (43). If RNase H acts as an endonuclease, it will convert the intact covalently closed Col E1 DNA (4.2×10^6 daltons) sedimenting at 23*S* to a mixture of single-stranded linear and circular molecules sedimenting at 17*S*. The sedimentation profile of covalently closed circles will not be affected if the RNase H acts only as an exonuclease. Figure 4 shows the sedimentation profiles of unreacted Col E1 DNA (panel A) and after incubation with M-MuLV DNA polymerase (panel B) and *E. coli* RNase H (panel C). Incubation with M-MuLV DNA polymerase did not convert the 23*S* covalently closed circular form of Col E1 DNA to a 17*S* form, whereas incubation with *E. coli* RNase H (known to be an endonuclease; 18) converted over 60% of the Col E1 DNA molecules from 23*S* to 17*S* forms. Alkali treatment or digestion with RNase A of Col E1 DNA also converted 60% of the molecules to 17*S* forms, indicating that a maximum of 60% of the DNA molecules contained ribonucleotides. The *E. coli* RNase H was free of DNase activity (M. J. Rosenfeld, personal communication). Thus, based upon these results, M-MuLV DNA polymerase-associated RNase H act as an exonuclease.

(ii) Is the enzyme a processive or random exonuclease? A processive nuclease is defined as an enzyme which, once bound to the polynucleotide chain substrate, completely degrades the substrate before being released. Thus, if excess [^{3}H]poly(A)·poly(dT) is incubated with a limiting amount of RNase H so that all the enzyme is bound, the addition of unlabeled poly(A)·poly(dT) to the incubation mixture should have no effect on the degradation of [^{3}H]poly(A) if the enzyme acts as a processive exonuclease. If, however, the enzyme acts as a random exonuclease, then the addition of excess unlabeled poly(A)·poly(dT) should retard the rate of degradation of [^{3}H]poly(A). Addition of

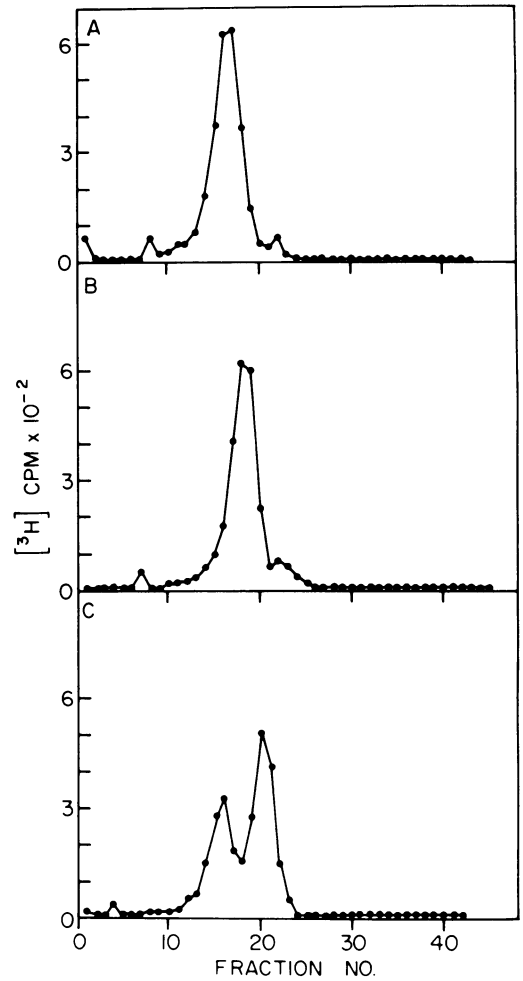

FIG. 4. *Sucrose gradient profiles of Col E1 DNA. 280 pmol of Col E1 DNA (30,000 counts/min per μg, average ribonucleotide chain length 25 nucleotides) was incubated in standard RNase H assay mixture with 20 U of M-MuLV DNA polymerase or E. coli RNase H for 60 min at 37 C. The amount of E. coli RNase H was equal to the amount of M-MuLV DNA polymerase-associated RNase H as defined by the amount of [^{3}H]poly(A) degraded from the [^{3}H]poly(A)·poly(dT)hybrid. The reaction was stopped by addition of 20 mM EDTA and the mixture was layered on sucrose gradients. (A) Control Col E1 DNA; (B) Col E1 DNA + M-MuLV DNA polymerase; (C) Col E1 DNA + E. coli RNase H.*

30-fold excess poly(A)·poly(dT) to the reaction mixture containing M-MuLV DNA polymerase 3 min after the start of incubation markedly retarded the subsequent release of acid-soluble radioactivity (Fig. 5A). Addition of excess poly(A)·poly(dT) to the reaction mixture before incubation also retarded the digestion of [^{3}H]-poly(A). Addition of 44-fold excess poly(A)-·poly(dT) to a reaction mixture containing AMV DNA polymerase 1 min after the start of incubation did not affect the release of acid-soluble radioactivity (Fig. 5B). The degradation of [^{3}H]poly(A)·poly(dT) was retarded if excess poly(A)·poly(dT) was added before incubation. The results confirm the processive nature of RNase H associated with AMV DNA polymerase that was reported previously (14, 21). In contrast to AMV DNA polymerase, RNase H associated with M-MuLV DNA polymerase acted predominantly as a random exonuclease.

(iii) What is the nature of the digested product? To test whether M-MuLV-associated RNase H digestion products have a 5'-phosphate and 3'-OH end, we performed the following two experiments. (i) Snake venom phosphodiesterase converted over 95% of the RNase H digested products to 5'-mononucleotides (Fig. 6A). Snake venom phosphodiesterase is a 3',5'-exonuclease; it requires a 3'-OH end for enzymatic activity and generates mononucleotides with a 5'-phosphate (7, 27). Less than 2% of radioactivity migrated with adenosine, suggesting that snake venom phosphodiesterase did not have significant phosphatase activity and hence eliminating the possibility that the 3' end of the product was first dephosphorylated and then digested. (ii) Alkaline hydrolysis of the

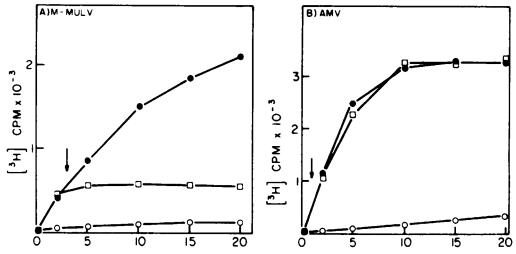

FIG. 5. *Effect of addition of excess poly(A)·poly(dT) on the rate of degradation of [^{3}H]poly(A)·poly(dT) by M-MuLV DNA polymerase and AMV DNA polymerase. (A) 30 pmol of [^{3}H]poly(A), 20 pmol of poly(dT), and 3.65 U of M-MuLV DNA polymerase were added to a standard reaction mixture. Unlabeled poly(A) (900 pmol) and poly(dT) (600 pmol) were added where indicated by arrow. 15-μl samples were withdrawn at various time intervals and acid-soluble radioactivity was determined. (B) 30 pmol of [^{3}H]poly(A), 2 pmol of poly(dT), and 9 U of AMV DNA polymerase were added to a standard reaction mixture containing 10 mM Mg^{2+} instead of Mn^{2+}. Unlabeled poly(A) (1,320 pmol) and poly(dT) (800 pmol) were added where indicated by arrow. 15-μl fractions were withdrawn at various time intervals and acid-soluble radioactivity was determined. Symbols: (●) Control; (□) excess substrate added after the start of incubation; (○) excess substrate added before the start of incubation.*

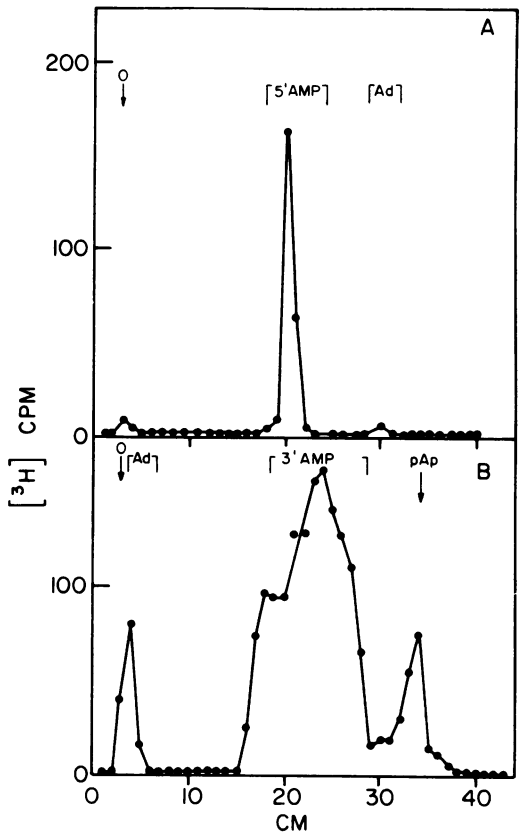

FIG. 6. *Nature of the digested product. 150 pmol of [^{3}H]poly(A) and 105 pmol of poly(dT) were incubated for 60 min with 30 U of M-MuLV DNA polymerase in a standard reaction mixture. The reaction was stopped by addition of trichloroacetic acid, and acid-soluble radioactive material was collected. (A) A portion of the acid-soluble material was neutralized with alkali, and 1 M glycine (pH 9.0) was added to a final concentration of 100 mM. 1 U of snake venom phosphodiesterase (in 0.01 M, pH 7.5) was added and incubated for 45 min at 37 C. 60 μg of 5'-AMP and 50 μg of adenosine were added as standards. Chromatography was performed for 22 h. (B) Another portion of acid-soluble material was boiled for 5 min in 0.3 N KOH and neutralized with 20% perchloric acid. The supernatant was removed by centrifugation (37) and applied to Whatman no. 1 filter paper. 50 μg of 3'-AMP was included as standard. Electrophoresis was performed from negative to positive.*

RNase H digested product and its subsequent electrophoresis showed that radioactivity migrated with adenosine, Ap, and pAp (Fig. 6B). The presence of pAp was detected by comparing its mobility with 3'-AMP (23). The amount of radioactivity migrating with adenosine was approximately equal to the radioactivity moving with pAp. Adenosine and pAp could have been generated only if the digestion product had a 5'-phosphate and a 3'-OH end. Based on the combined results with snake venom phosphodiesterase and alkaline hydrolysis of the digested product, it can be concluded that M-MuLV DNA polymerase-associated RNase H cleaves at the 3' side of the 3',5'-phosphodiester bond to yield products containing 5'-phosphate and 3'-OH ends.

(iv) What is the size of the product? [^{3}H]poly(A)·poly(dT) was digested with M-MuLV DNA polymerase and AMV DNA polymerase to convert over 85% of the [^{3}H]poly(A) to an acid-soluble form. The size of the digested products was analyzed by paper chromatography (Fig. 7). Over 90% of the radioactivity remained at the origin [larger than $(A)_9A$] in the material digested by M-MuLV DNA polymerase, and 70% of the radioactivity was retained at the origin in the material digested by AMV DNA polymerase (Table 6). Material migrating from the origin had heptamers, pentamers, and trimers, but no monomer could be detected.

Polyacrylamide gel electrophoresis of acid-soluble digested products revealed that M-MuLV DNA polymerase digested material was larger in size than the AMV DNA polymerase digested material (data not shown). The average size of the M-MuLV DNA polymerase digested product was 15 to 20 nucleotides, whereas AMV DNA polymerase digestion products had an average size of 10 to 12 nucleotides.

DISCUSSION

M-MuLV DNA polymerase can transcribe both ribohomopolymers and deoxyribohomopolymers. Purified virions of M-MuLV, when incubated with deoxyribonucleoside triphosphates and detergent, can synthesize complementary DNA as efficiently as purified avian myeloblastosis virions (39). However, 70*S* viral RNA is less efficiently transcribed by purified M-MuLV DNA polymerase than by purified AMV DNA polymerase. If, however, oligo(dT) is added as a primer, the efficiencies of transcription by the two enzymes are comparable. M-MuLV DNA polymerase can utilize 10*S* globin mRNA·oligo(dT) as template-primer to synthesize complementary DNA in the presence or absence of actinomycin D. The complementary DNA synthesized in the presence of actinomycin D (added to prevent the synthesis of double-stranded DNA) by either M-MuLV DNA polymerase or AMV DNA polymerase is 680 nucleotides long (Hpa II digested fragments 6, 7, and 8 of polyoma DNA, kindly provided by M. Vogt, were used as standards; data not shown). The complementary DNA synthesized by M-MuLV DNA polymerase in the absence of actinomycin D is double stranded (over 90% resistant to S1

TABLE 6. *Distribution of RNase H digestion products*[a]

Enzyme	% acid soluble	% radioactivity in digestion products	
		$\geq(pA)_{10}$	$\leq(pA)_{10}$
M-MuLV	>85	90	10
AMV	>90	70	30

[a] Details of the reaction are described in the legend to Fig. 5. The data are an average of five experiments.

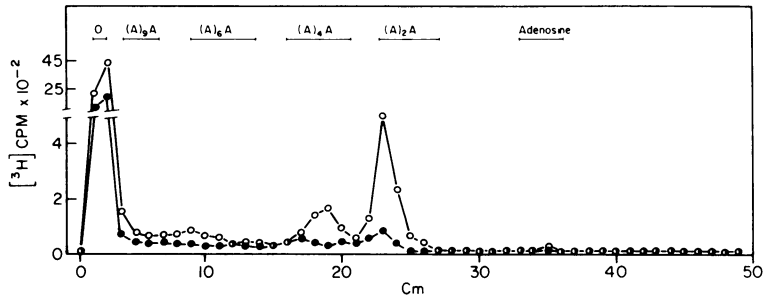

FIG. 7. *Chromatography of [^{3}H]poly(A) digestion products. A portion of acid-soluble material obtained from digestion of [^{3}H]poly(A)·poly(dT) by M-MuLV DNA polymerase (see legend to Fig. 5) and acid-soluble material obtained from digestion by AMV DNA polymerase was chromatographed for 22 h. 50 to 100 μg of $(A)_9A$, $(A)_6A$, $(A)_4A$, and $(A)_2A$, and adenosine were included as standards. The position of the standards was located by shining UV light. Symbols: (●) M-MuLV DNA polymerase; (○) AMV DNA polymerase.*

nuclease digestion), suggesting that it can synthesize double-stranded DNA from single-stranded DNA.

M-MuLV DNA polymerase and the α subunit of AMV DNA polymerase share some common features; (i) the presence of template-primer does not protect the enzyme from heat inactivation under the conditions where the $\alpha\beta$ complex of AMV DNA polymerase is completely protected, and (ii) the α subunit (14) and the M-MuLV DNA polymerase are both random exonucleases, whereas the $\alpha\beta$ complex is a processive exonuclease (14, 21). It is possible that M-MuLV DNA polymerase has also two subunits, but one of them is lost during the enzyme purification. The putative second subunit may be necessary for the efficient transcription of 70*S* viral RNA.

There are some possibilities that may explain the difficulties encountered in the detection of RNase H activity associated with purified MuLV DNA polymerase.

(i) The large size of the digestion product. The criteria used for the detection of RNase H activity are to monitor either a decrease of radioactivity in acid-insoluble material or an increase in radioactivity in acid-soluble material. The large size of digested product may prevent it from dissociating from the DNA or, alternatively, the digested product may not be acid soluble.

(ii) The conditions of the reaction, particularly the choice of the divalent cation. Mn^{2+} is preferred over Mg^{2+}.

(iii) Certain strains of murine leukemia viruses do not have RNase H-associated activity.

DNA polymerase manifesting RNase H activity from avian RNA tumor viruses is coded for by the viral RNA (34, 36). The presence of RNase H activity associated with M-MuLV DNA polymerase suggests that the nuclease activity may be an integral part of DNA polymerase from all RNA tumor viruses. It is not clear what function a random exonuclease that cleaves after every 10 or 15 nucleotides would play in the growth cycle of RNA tumor viruses. It is perhaps significant that the digestion product has a 3'-OH end that could serve as a primer for the synthesis of second strand (4). However, addition of sodium fluoride (12 mM), which specifically inhibits RNase H activity (8), does not affect the synthesis of double-stranded DNA (Verma, unpublished data). Furthermore, when double-stranded DNA was synthesized with α-^{32}P-labeled deoxyribonucleoside triphosphates, no transfer of radioactivity to ribonucleotides was observed (34; Verma, unpublished data). Since the provirus has been shown to be double-stranded DNA (33), RNase H may be required to remove the template 70*S* RNA from the 70*S* RNA single-stranded complementary DNA hybrid to allow the synthesis of double-stranded DNA.

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ADDENDUM IN PROOF

In a recent report, K. Mölley (Virology **62**:46–59, 1974) has shown that DNA polymerase from Friend murine leukemia virus has RNase H activity.

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