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DNA Polymerase of Murine Sarcoma-Leukemia Virus: Lack of Detectable RNase H and Low Activity With Viral RNA and Natural DNA Templates

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Kirsten murine sarcoma-leukemia virus (Ki-MSV[MLV]) was found to contain less RNase H per unit of viral DNA polymerase than avian Rous sarcoma virus (RSV). Upon purification by chromatography on Sephadex G-200 and subsequent glycerol gradient sedimentation the avian DNA polymerase was obtained in association with a constant amount of RNase H. By contrast, equally purified DNA polymerase of Ki-MSV(MLV) and Moloney [Mo-MSV(MLV)] lacked detectable RNase H if assayed with two homopolymer and phage *fd* DNA-RNA hybrids as substrates. On the basis of picomoles of nucleotides turned over, the ratio of RNase H to purified avian DNA polymerase was 1:20 and that of RNase H to purified murine DNA polymerase ranged between $<1:2,800$ and 5,000. Based on the same activity with poly (A)·oligo(dT) the activity of the murine DNA polymerase was 6 to 60 times lower than that of the avian enzyme with denatured salmon DNA template or with avian or murine viral RNA templates assayed under various conditions (native, heat-dissociated, with or without oligo(dT) and oligo(dC) and at different template enzyme ratios). The template activities of Ki-MSV(MLV) RNA and RSV RNA were enhanced uniformly by oligo(dT) but oligo(dC) was much less efficient in enhancing the activity of MSV(MLV) RNA than that of RSV RNA. It was concluded that the purified DNA polymerase of Ki-MSV(MLV) differs from that of Rous sarcoma virus in its lack of detectable RNase H and in its low capacity to transcribe viral RNA and denatured salmon DNA. Some aspects of these results are discussed.

Ribonuclease H (RNase H), an enzyme that degrades RNA only if based paired with DNA (17, 25), has been shown to be an integral part of the DNA polymerase of several avian leukosis (1, 19, 20, 24, 29) and sarcoma (unpublished observations, 1972) viruses. Therefore, it is thought that this RNase H plays an essential role in the transcription of viral RNA to DNA during virus replication (19, 20, 24). In addition, variable amounts of free, presumably cellular RNase H were found in avian myeloblastosis virus (W. Keller, 1972, personal communication).

RNase H has also been found associated with other classes of RNA tumor viruses including murine and feline viruses (13), and it has been claimed that the RNase H of at least one mammalian RNA tumor virus, Moloney murine sarcoma-leukemia virus, is an integral part of the viral DNA polymerase (13).

The studies reported here were done to test whether the DNA polymerase of murine sarcoma-leukemia virus (MSV[MLV]) is free or associated with viral RNase H. It was found that no RNase H activity could be detected in the purified DNA polymerase of Kirsten virus. Under identical conditions RNase H activity was readily detected in purified DNA polymerase of Rous sarcoma virus (RSV). Further, it was found that compared to its activity with poly(A)·oligo(dT) the murine enzyme has a 6- to 60-fold lower template activity with viral RNA and salmon DNA than the avian DNA polymerase.

MATERIALS AND METHODS

Reagents. The following reagents were purchased: unlabeled deoxyribonucleosidetriphosphates and salmon sperm DNA from Sigma Chemical Co.; dithiothreitol (DTT) from Calbiochem; Triton X-100 from

Packard Instruments; bovine serum albumin (BSA) from Schwarz/Mann Research or Miles Laboratories; poly(A)·oligo(dT) (base ratios 1:1) from Collaborative Research Inc., and poly(dG)(S_{w20} = 4.4) from P-L Biochemicals Inc.; poly(dT)(S_{w20} = 2.3); ^{3}H-poly(A) (S_{w20} = 12.4, 85.6 Ci/M phosphate); ^{3}H-poly-(C) (mol. wt. 50,000, 44.8 Ci/M phosphate) from Miles Laboratories Inc.; ^{3}H-TTP (18 Ci/mM) and ^{3}H-dCTP (25 Ci/mM) from New England Nuclear Corp.; Oligo(dT)$_{12-18}$ and oligo(dC)$_{12-18}$ from Collaborative Res. Inc., Waltham, Mass., and gentamicin from Microbiol. Assoc., Inc.

Buffer solutions. Standard buffer contained 0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 7.2) and 1 mM ethylenediaminetetraacetate (EDTA). Buffer A is 0.01 M Tris-hydrochloride pH 8.0, 2 mM EDTA, 1 mM DTT, and 0.1 M KCl. Buffer B contained 0.4 M NaCl, 0.015 M Na-phosphate buffer (pH 6.8), 0.2% β-mercaptoethanol, 0.2% Triton X-100, and 30% glycerol.

Virus. Prague RSV of subgroup C (PR RSV-C), a recombinant of PR RSV of subgroup A and Rous associated virus of subgroup B (RAV-2) termed PR RSV-B (obtained from Peter K. Vogt, USC, Los Angeles) were propagated and purified according to published procedures (6, 7). Ki-MSV(MLV) was harvested at daily intervals from a transformed rat kidney (NRK) cell line grown in Eagle minimal essential medium supplemented with 10% fetal calf serum, 1% dimethylsulfoxide, 0.5 μg of fungazone per ml; 100 U of penicillin per ml; 50 μg of streptomycin per ml and 25 μg of gentamicin per ml (22). Confluent cultures were maintained in the above medium containing only 5% serum. Moloney strain of mouse leukemia virus (Mo-MLV) (obtained from A. J. Hackett, UC Berkeley, Oakland, Naval Biol. Lab.) was propagated by infecting the high-passage mouse embryo cell line (HPME) (3) and harvested similarly as described for Ki-MSV(MLV). Mo-MSV(MLV) was propagated in a mouse cell line (SC-1) partially transformed by the virus and obtained from W. P. Rowe, NCI, Bethesda, Md. Rauscher mouse leukemia virus (Ra-MLV) was obtained from Electro-Nucleonics Laboratory, Bethesda, Md. The purification of murine viruses was as described for avian tumor viruses. Purified virus was stored at −20 C in standard buffer containing 50% glycerol. The half-life of viral enzymatic activities in this condition was over one month. The A_{260} of purified viruses was determined in standard buffer containing 0.2% Na dodecyl sulfate.

Preparation of 50 to 70*S* and ^{3}H-labeled 4 to 12*S* viral RNA followed published procedures (2, 4, 6, 7).

Polymerase assay. The standard assay using poly(A)·oligo(dT) as template was done in 100 μliters containing 50 mM Tris-hydrochloride (pH 8.1), 50 mM KCl, 6 mM $Mg(Ac)_2$, 5 mM DTT, 2.5 μM ^{3}H-TTP, 150 μM TTP, and 2 μg of poly(A)·oligo(dT). The specific radioactivity of TTP in this assay was 200 counts per min per pmol.

Assays using 50 to 70*S* viral RNA or denatured salmon sperm DNA as templates were done in 100 μliters containing 0.1 mM dNTP, 2.5 μM ^{3}H-TTP or ^{3}H-dCTP, 1 or 2 μg of viral RNA or 2.5 μg KOH denatured salmon sperm DNA (8, 9) under the same conditions as for the poly(A)·oligo(dT) assay. All assays were at 37 C for 60 or 120 min as indicated. Determination of reaction products was described (8, 9). The specific radioactivity in these assays was 12,500 counts per min per pmol for ^{3}H-TTP or 17,000 counts per min per pmol for ^{3}H-dCTP.

RNase H assay. Assay I. The standard assay was done in 100 μliters of solution containing 50 mM Tris-hydrochloride (pH 7.8), 20 mM $Mg(Ac)_2$, 10 mM DTT, and either 215 pmol of ^{3}H-poly(A) (60 counts per min per pmol) plus 110 pmol of poly(dT), or 250 pmol of ^{3}H-poly(C) (~32 counts per min per pmol) plus 125 pmol of poly(dG). Assays were for 60 or 120 min at 37 C. Thereafter, EDTA was added to stop the reaction. Solubilized ^{3}H-poly(A) or ^{3}H-poly(C) was calculated by subtracting the remaining 5% trichloroacetic acid-precipitable radioactive homopolymer collected on membrane filters (0.45 μm pore size; Millipore Corp.) from the total acid-precipitable radioactive homopolymer incubated under the same conditions without enzyme.

Assay II. Some assays were done in 100 μliters containing 30 mM Tris-hydrochloride (pH 7.8), 0.1 M $(NH_4)_2SO_4$; 2 mM Mn Cl_2, and the same amount of ^{3}H-poly(A)·poly(dT) as described above. All other conditions were the same as in assay I.

Enzyme units. The units of RNase H and DNA polymerase were defined arbitrarily as follows: One unit of DNA polymerase represents the amount of enzyme that catalyzes incorporation of 100 pmol (TMP) per h at 37 C by using poly(A)·oligo(dT) as template under standard assay conditions. One unit of RNase H represents the amount of enzyme that hydrolyzes 100 pmol (AMP) per h at 37 C by using ^{3}H-poly(A)·poly(dT) as substrate in the conditions described for assay I.

Solubilization of enzymes. Purified virus in standard buffer containing about 50% glycerol was mixed with two volumes of buffer A and a 10% solution of Triton X-100 was added to a final concentration of 0.5%. After incubation at 37 C for 15 min the mixture was centrifuged at 100,000 × *g* in the type 40 Spinco rotor for 15 min at 20 C. The enzymes in the supernatant fluid are referred to as soluble enzymes and were directly used for further analyses. In some cases the supernatant fluid containing the soluble enzymes of avian, but not of murine tumor viruses was subjected to $(NH_4)_2SO_4$ precipitation (45%) and then was resuspended in buffer A or buffer B. In recent experiments the enzymes of MSV(MLV) were solubilized at 0 C in order to retain maximal enzymatic activity.

Sephadex chromatography. Sephadex G-200 (Pharmacia, Fine Chemicals, Inc.) was allowed to swell at 100 C for 5 h in buffer B and packed into a column (2.5 by 80 cm). Enzyme was eluted with the same buffer at a rate of 3 to 4 ml per h. All procedures except the swelling of gel were performed at 4 C (10).

RESULTS

RNase H activity in purified avian and murine RNA tumor viruses. It is shown in Table 1 that there is less RNase H in murine RNA tumor viruses than in avian tumor viruses.

Based on a unit of viral DNA polymerase (Table 1) the murine viruses tested contain only 5 to 20% of the RNase H found in avian viruses. If RNase H activities per A_{260} of purified viruses are compared, the murine viruses also contain less (5 to 40%) RNase H than the two avian viruses investigated. The variability of the RNase H/DNA polymerase ratios of different viruses within the avian or murine group is probably due to different degrees of purity and integrity of the virus preparations compared.

RNase H activity is copurified with the DNA polymerase of avian but not of murine tumor viruses: (i) RNase H activity and DNA polymerase of RSV. Chromatographic analysis on Sephadex G-200 and subsequent sedimentation of the soluble (Materials and Methods) enzymes of RSV are shown in Fig. 1A and B. The DNA polymerase activity formed a single peak under both conditions. It coincided with over 50% of the total virus-associated RNase H after chromatography (Fig. 1A), and the ratio of RNase H units to DNA polymerase units varied between 0.04 and 0.055 across the DNA polymerase peak. Upon further analysis of this peak by glycerol gradient sedimentation [fractions 61 to 71, concentrated by 45% $(NH_4)_2SO_4$] both enzymes coincided as symmetrical peaks. The ratio of RNase H to DNA polymerase was not significantly affected by velocity sedimentation and remained at 0.05 (Fig. 1B).

Some RNase H did not cochromatograph with viral DNA polymerase in Fig. 1A. It consists of RNase H with apparent high (fractions 44 to 52) and low molecular weight (all fractions over no. 71). This RNase H was not associated with viral DNA polymerase and may be cellular RNase H.

We conclude that most of the RNase H of RSV is linked to viral DNA polymerase and that the two activities appear to reside on the same enzyme or enzyme complex. This is in accord with earlier observations on an avian leukosis virus (1, 19, 20, 24, 29). Moreover, the enzymatic activities prepared in this fashion were practically free of detectable RNase degrading single-stranded RNA. If purified 30 to 40S RSV RNA subunits were incubated for 30 min with the enzyme at 40 C in polymerase or RNase H assay conditions (Materials and Methods) no detectable degradation was observed after anal-

TABLE 1. *Comparison of DNA polymerase and of RNase H in avian and murine RNA tumor viruses*

Virus	DNA polymerase:[a] A_{260} virus[b]	RNase H:[a] A_{260} virus	RNase H: DNA polymerase
PR RSV-C[c]	46	6	0.13
PR RSV-B	538	38	0.07
Ki-MSV(MLV)[c]	290	2	0.007
Mo-MLV	315	2	0.0064
Ra-MLV	350	5	0.0145

[a] The numbers represent units of DNA polymerase and RNase H as defined in Materials and Methods.

[b] One A_{260} unit of purified virus measured in standard buffer in the presence of 0.2% Na dodecylsulfate (Materials and Methods).

[c] Average of three different determinations.

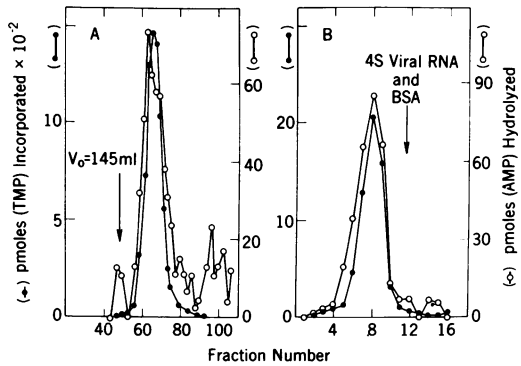

FIG. 1. *Chromatography on Sephadex G200 and subsequent glycerol gradient sedimentation of the DNA polymerase and the RNase H of RSV. A, The soluble enzymes of 40 A_{260} units of purified PR RSV-C were prepared in total volume of 9 ml as described in Materials and Methods. The enzyme solution was then mixed at 4 C with a proper volume of a saturated solution of $(NH_4)_2SO_4$ to make a final concentration of 45%. The precipitate (100,000 × g, 15 min, 5 C) was redissolved in 2 ml of buffer B and applied to a Sephadex G200 column. Elution was at a rate of 3 ml per h at 5 C (see Materials and Methods). Fractions (3 ml) were collected. A 15-μliter sample of every second or third fraction was assayed for DNA polymerase and a 25-μliter sample for RNase H activity. Assays were for 2 h as described in Materials and Methods. Vo is the void volume of our Sephadex column. B, Peak fractions nos. 61 to 71 shown in Fig. 1A were pooled. Saturated $(NH_4)_2SO_4$ and 10% Triton X-100 were added to a final concentration of 45% and 0.4%, respectively, at 4 C; after gentle stirring the solution was incubated in an ice bath for 15 min. It was then centrifuged as described for Fig. 1A and the precipitate was resuspended in 0.4 ml of buffer A. The enzyme solution was then applied to a 5-ml 20 to 40% glycerol gradient containing 10 mM Tris-hydrochloride (pH 8.1), 0.1 M KCl, 1 mM EDTA, 0.2 mM DTT, 0.2% Triton X-100, and centrifuged in Spinco SW 65 rotor at 64,000 rpm for 9 h at 15 C. Twelve-drop (0.36 ml)-fractions were collected. A 15-μliter sample of each fraction was assayed for DNA polymerase and a 25-μliter sample for RNase H at 37 C for 2 h.*

ysis by formaldehyde (4) sucrose grâdient sedimentation (unpublished data).

(ii) RNase H activity and DNA polymerase of murine leukemia and sarcoma viruses. Chromatographic analysis on Sephadex G-200 and subsequent sedimentation of the soluble (Materials and Methods) enzymes of Ki-MSV(MLV) are shown in Fig. 2A and B. No detectable RNase H activity cochromatographed with the peak of viral DNA polymerase. The limit of RNase H detection in the peak fraction (no. 66, Fig. 2A) of viral DNA polymerase was about 1 to 2 pmol (≈60 to 120 counts/min) of A hydrolyzed from poly(A) per 600 pmol of dTMP incorporated by DNA polymerase in response to poly(A)·oligo(dT). By contrast, the same amount of avian DNA polymerase (peak fraction no. 62, Fig. 1A) hydrolyzed about 30 pmol of A from poly(A). All soluble RNase H from Ki-MSV(MLV) eluted with a rather irregular distribution in the low molecular weight region of the column (Fig. 2A).

The DNA polymerase peak of Ki-MSV(MLV) (Fig. 2A, fractions 65 to 72) was then concentrated 20-fold by dehydration with dry Sephadex G-200. Precipitation of the DNA polymerase of Ki-MSV(MLV) with $(NH_4)_2SO_4$ (Schwarz/Mann Co., N. Y.) was not possible because this inactivated the polymerase either largely, if unpurified enzyme was used, or completely, if partially purified enzyme was used. By contrast, Rauscher leukemia virus DNA polymerase was reported to be little affected by $(NH_4)_2SO_4$ precipitation (18).

The concentrated DNA polymerase was then fractionated by glycerol gradient sedimentation. It can be seen in Fig. 2B that the DNA polymerase formed a single peak and that the peak fraction (no. 10, Fig. 2B) incorporated 2,800 pmol of dTMP in response to poly(A)·oligo(dT) but hydrolyzed no detectable A from poly(A)·poly(dT) if assayed under two different conditions (e.g., assay I, Fig. 2A, or RNase H assay II, [Materials and Methods] not shown). It also failed to hydrolyze poly(C) if ^{3}H-poly(C)·poly(dG) was used as substrate (Fig. 2B). From some virus preparations a DNA polymerase without detectable RNase H could be obtained by gradient sedimentation only, without prior Sephadex G-200 chromatography (Table 3). This appeared to depend on the variable RNase H content of the virus preparations used.

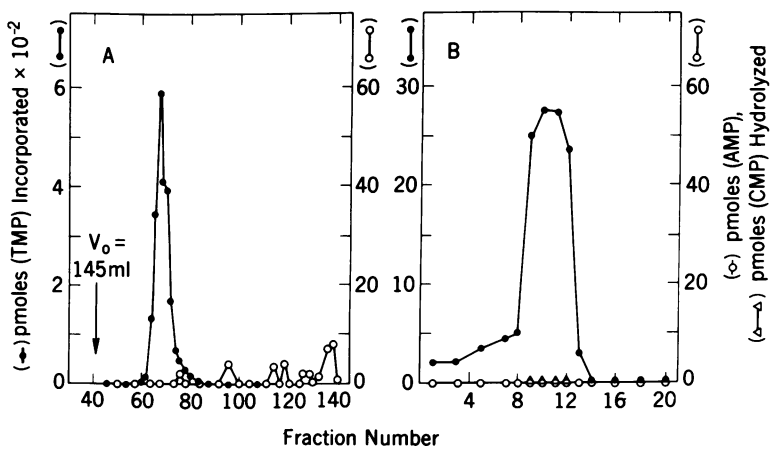

FIG. 2. *Chromatography on Sephadex G200 and subsequent glycerol gradient sedimentation of the DNA polymerase and the RNase H of Ki-MSV(MLV). A, Ten A_{260} units of Ki-MSV(MLV) virus in 1 ml of standard buffer containing about 50% glycerol were solubilized at 37 C for 15 min (see Materials and Methods). After centrifugation, the soluble enzymes were chromatographed as described for Fig. 1A. Fractions (3.3 ml) were collected. A 15-μliter amount of every 2nd (peak fractions) or 4th (others) fraction was assayed for DNA polymerase and a 25-μliter amount was assayed for RNase H. Assays were at 37 C for 2 h as described in Materials and Methods. B, Peak fractions nos. 65 to 72 (Fig. 2A) were pooled and transferred into a dialysis tube. The dialysis tube was kept in contact with dry Sephadex G200 at 4 C for 3 days until the volume was reduced about 20-fold. Recovery of enzyme activity was about 50%. The concentrated enzyme preparation was then layered on a 12-ml 20 to 40% buffered glycerol gradient as described for Fig. 1B. The gradient was poured on top of a cushion of 0.2 ml 85% glycerol containing the same buffer as the gradient. Sedimentation was in a Spinco SW 41 rotor at 40,000 rpm for 43.5 h at 15 C. Fractions (0.6 ml) were collected. Samples (50 μliters) of each fraction were assayed for DNA polymerase by using poly(A)·oligo(dT) and RNase H activity by using ^{3}H-poly(A)·poly(dT) or ^{3}H-poly(C)·poly(dG) in conditions described for assay I (see Materials and Methods).*

Our failure to detect RNase H activity with homopolymer substrates in purified DNA polymerase of Ki-MSV(MLV) does not exclude that the enzyme contains a RNase H activity with another substrate specificity. For example, it was shown that bacterial RNase H digests poly(dA)·poly(U) whereas viral RNase H does not (20). Since natural DNA-RNA hybrids presumably are the least discriminating substrates for RNase H activities of different specificities, we have tested the purified DNA polymerases of RSV, Ki-MSV(MLV) and Mo-MSV(MLV) with a phage *fd* DNA-RNA hybrid. Both the RNA and the DNA moieties of this hybrid were labeled with isotopes. It is shown in Table 2 that purified RSV DNA polymerase degraded most of the RNA of *fd* DNA-RNA as well as of poly(A)·poly(dT) hybrids, whereas the purified DNA polymerases of the two murine sarcoma-leukemia viruses did not. The RNA of *fd* DNA-RNA was much more resistant to RNase H of PR RSV-B after heat dissociation than before. By contrast the RNA of the hybrid was resistant to RNase A and RNase T1 before but not after heat dissociation (Table 2).

We conclude that the purified DNA polymerases of Ki- and Mo-MSV(MLV) do not contain detectable RNase H for the natural *fd* DNA-RNA hybrid. Further, the avian and in particular the murine DNA polymerases tested contain very little, if any, DNase activity that renders *fd* DNA acid soluble.

(iii) Comparison of the RNase H activity associated with avian and murine DNA polymerase. The ratios of picomoles of A hydrolyzed of a poly(A)·poly(dT) substrate per picomole of dTMP incorporated in response to poly(A)·oligo(dT) for different viral enzyme preparations are summarized in Table 3. It is shown that the purified DNA polymerases of different strains of RSV have under our conditions an RNase H to DNA polymerase ratio which on a basis of picomoles turned over may be expressed as 1:20. Using the same methods for comparison different preparations of DNA polymerase of Ki-MSV(MLV) had a RNase H to DNA polymerase ratio ranging from $<1:240$ to $<1:5{,}000$. That is to say, in most preparations of Ki-MSV(MLV) DNA polymerase, RNase H activity was not detectable by our methods. We conclude that the DNA polymerase of Ki-MSV(MLV) contains at least 100 to 200 times less RNase H activity than the avian counterpart and may perhaps lack this enzyme all together. The absence of RNase H appeared not to be due to the presence of an unspecific inhibitor, because the RNase H activity of RSV DNA polymerase was not affected if purified DNA polymerase of Ki-MSV(MLV) was added (Table 3).

Estimation of the molecular weights of RSV- and MSV(MLV)-DNA polymerase. Based on a 4*S* bovine serum albumin (BSA) standard (16) or a 4*S* viral RNA standard (2, 4), both analyzed in parallel rotor tubes, the sedi-

TABLE 2. *Effects of RSV, Ki-MSV(MLV), and Mo-MSV(MLV) DNA polymerases on DNA-RNA hybrids*

Enzyme source	Purification	*fd* ^{3}H-DNA·^{32}P-RNA[a]				^{3}H poly(A)·poly(dT) (% poly(A) digested)	Polymerase activity (units)
		Native		Denatured[b]			
		DNA	RNA	DNA	RNA		
PR-RSV-B	Gradient	10.0[c]	75.5			69.1	32.8
	G-200 + gradient	6.5	80.0	0	33.7	60.0	21.5
Ki-MSV(MLV)	Gradient	0	2.5			0.5	45.5
	G-200 + gradient	1.8	2.4	0	4.5	1.6	40.0
PR-RSV-B + Ki-MSV(MLV)	Gradient	4.2	82.6			60.0	60.0
Mo-MSV(MLV)	Gradient	0	4.7			0	42.5
RNase A (5 μg/ml) + RNase T_1 (150 U/ml)[d]		0	0	2	82.6		

[a] *fd* ^{3}H-DNA·^{32}P-RNA hybrid was made using single-stranded *fd* ^{3}H-DNA circles as templates, ^{32}P-CTP as labeled nucleotide and *E. coli* RNA polymerase according to the procedure of Berg et al. (1a). The specific activities of ^{3}H-DNA were 2,400 counts per min per nmol and of ^{32}P-RNA were 5,000 counts per min per nmol of nucleotide. Hybrid containing 0.83 nmol DNA (≃2,000 counts/min) and 0.4 nmol RNA (≃2,000 counts/min) was used per 2-h assay; otherwise the conditions were the same as described in Materials and Methods.

[b] DNA-RNA hybrid was denatured by heating at 100 C for 2 min in standard buffer but containing only 10 mM NaCl (Materials and Methods).

[c] Percent digested.

[d] Both RNases were purchased from Worthington Biochemical Corp. RNase A was preincubated at 100 C for 5 min to inactivate DNase.

TABLE 3 *RNase H activity in purified viral DNA polymerases*

Virus source	Method of purification	RNase H[a]: DNA polymerase[b]
PR RSV-B	Glycerol gradient	1:23
PR RSV-C	Glycerol gradient	1:18
PR RSV-C	Sephadex G200 + glycerol gradient	1:23
Ki-MSV(MLV)	(exp. 1) Glycerol gradient	1:240
	(exp. 2) Glycerol gradient	1:210
	(exp. 3) Glycerol gradient	<1:4,000[c]
	(exp. 4) Glycerol gradient	<1:5,000[c]
	Sephadex G200 (Fig. 2A)	<1:600[c]
	(exp. 1) Sephadex G200 + glycerol gradient	<1:2,800[c]
	(exp. 2) Sephadex G200 + glycerol gradient	<1:2,900[c]
Ki-MSV(MLV)[d] + PR RSV-B	Glycerol gradient	1:41

[a] The numbers represent pmoles of AMP hydrolyzed from ^{3}H-poly(A)·poly(dT) during 1 h at 37 C as described in Materials and Methods.

[b] The numbers represent pmoles of dTMP incorporated in response to poly(A)·oligo(dT) during 1 h at 37 C under the conditions described in Materials and Methods.

[c] RNase H activity was too low to be detected by our assay.

[d] Units (20) of Ki-MSV(MLV) and PR RSV-B DNA polymerase were mixed.

mentation coefficient of the RSV DNA polymerase was estimated (23) to be 8*S* (Fig. 1B). The same result was obtained in two other independent experiments. By contrast the sedimentation coefficient of Ki-MSV(MLV) DNA polymerase was found to be 4.5*S* using the same method of determination and the same standards (Fig. 3 and Fig. 2B). Using BSA as a MW standard of 67,000 and the formula (Sw1/Sw2) = (MW1/MW 2)$^{2/3}$ to convert Sw to MW (23) we calculate the MW of RSV DNA polymerase to be 161,000 and that of MSV(MLV) DNA polymerase to be 70,000. The values agree with earlier determinations on RSV DNA polymer-

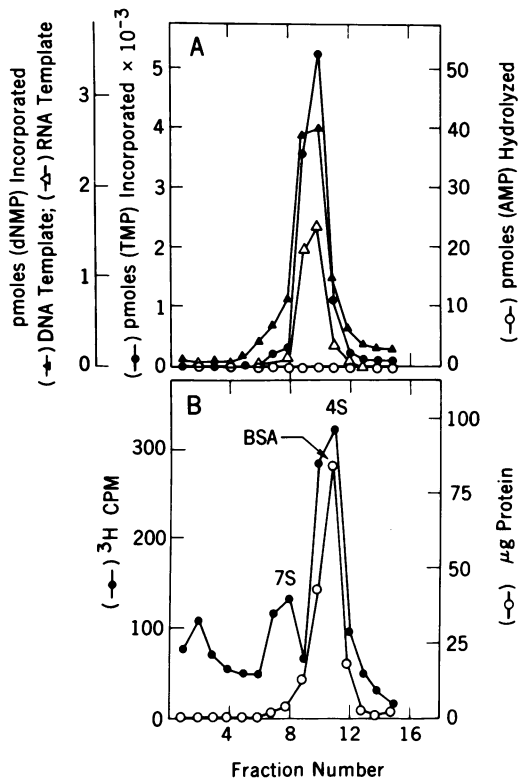

FIG. 3. *Sedimentation of Ki-MSV(MLV) DNA polymerase and of sedimentation standards. A, The enzymes of 1.5 A_{260} units of Ki-MSV(MLV) virus in 0.1 ml of standard buffer containing about 50% glycerol were solubilized (Materials and Methods) at 0 to 4 C for 20 min. After centrifugation the supernatant was applied to a 5-ml 20 to 40% glycerol gradient and sedimented at 65,000 rpm for 12 h at 5 C (as described for Fig. 1B). Twelve-drop (~0.36 ml) fractions were collected. Samples (25 μliters) of each fraction were assayed for DNA polymerase using poly(A)·oligo(dT), 55S Ki-MSV(MLV) RNA and salmon DNA as templates (Materials and Methods). RNase H was assayed on 25-μliter samples using conditions described for assay I in Materials and Methods. B, A gradient containing low molecular weight viral 4S and 7S (2) ^{3}H-RNA and 300 μg of BSA was centrifuged in a parallel tube. Fractions were collected as described in Fig. 3A. Samples (50-μliters) of each fraction were placed into 3 ml of toluene-based scintillation fluid containing 10% NCS (Nuclear Chicago) and radioactivity was determined in a TRICARB (Packard Co.) liquid scintillation counter. The remainder of each fraction was then precipitated with 70% ethanol to remove Triton X-100 and redissolved in 0.5 ml of water. Protein was then determined on each fraction by the Lowry method (21).*

ase (8) and on the DNA polymerase of Rauscher murine leukemia virus (12, 18, 27).

Comparing the template activities of poly(A)·oligo(dT), 50 to 70S viral RNA and denatured salmon DNA for murine and avian DNA polymerase. Preliminary experiments indicated that gradient purified MSV(MLV) DNA polymerase obtained by disrupting virus at 37 C had very little activity with 55S Ki-MSV(MLV) RNA (22), or denatured salmon DNA as template compared to its activity with poly(A)·oligo(dT). In order to retain maximal activity the DNA polymerase of MSV(MLV) was solubilized at 0 C and gradient purified at 5 C (Fig. 3). It was found that the peak activities of viral DNA polymerase with poly(A)·oligo(dT), denatured salmon DNA and 55S Ki-MSV(MLV) RNA coincided (Fig. 3). On the basis of picomoles nucleotide incorporated per 60 min at 37 C, the template activities of the three template-primers were 3,500:1.8:1, respectively. A different preparation of Kirsten DNA polymerase gave very similar results. The relative activities of the three template-primers were 3,000:1.9:1.5 (Table 4). The absolute activity of MSV(MLV) DNA polymerase recovered at 37 C and sedimented at 5 C was about 2.5 times lower than that recovered at 0 to 4 C but the relative template activities of the three templates compared were unchanged (data not shown).

The relative template activities of poly(A)·oligo(dT), salmon DNA and 60 to 70S RSV RNA for purified RSV DNA polymerase were about 3,000:48:28, under the same conditions (Table 4).

The failure of MSV(MLV) DNA polymerase to transcribe MSV(MLV) RNA efficiently could be due to defects of the viral RNA template or of the enzyme. To test whether the MSV(MLV) RNA lacks a primer or a specific template sequence essential for efficient transcription to DNA, the following types of experiments were performed: (i) The responses of RSV DNA polymerase to native 55S Ki-MSV(MLV) RNA and to 60 to 70S RSV RNA were tested in parallel. As seen in Table 4 and Fig. 4, the template activity of MSV(MLV) RNA was about the same as that of RSV RNA. However, the activity of Ki-MSV(MLV) RNA was about 15-fold and that of 60 to 70S RSV RNA about 20- to 40-fold lower with murine DNA polymerase, depending on the triphosphate used (Fig. 4), than with RSV DNA polymerase (Table 4 and Fig. 4). In template saturation experiments (Fig. 4) it was found that, at the concentrations used in Table 4, the RNAs had almost maximal activities with the respective enzymes. With MSV (MLV) DNA polymerase both MSV (MLV) RNA and RSV RNA showed optimal template activity at 0.5 to 2 μg/0.1 ml and had lesser activities at higher or lower concentrations in our conditions (Fig. 4B). The same was true if MSV(MLV) RNA and tobacco mosaic virus RNA were tested with RSV DNA polymerase (Fig. 4A). By contrast raising the concentration of RSV RNA up to 8 μg/0.1 ml did not reduce DNA synthesis with the RSV en-

TABLE 4. *Response of PR RSV-B and Ki-MSV(MLV) DNA polymerases to various template-primers*[a]

Enzyme source	RSV RNA[b]						MSV(MLV) RNA[b]						Salmon sperm DNA	Poly(A)·oligo(dT)
	Native			Heated[c]			Native			Heated				
	−	+dT_{12-18}[d]	+dC_{12-18}[d]	−	+dT_{12-18}	+dC_{12-18}	−	+dT_{12-18}	+dC_{12-18}	−	+dT_{12-18}	+dC_{12-18}		
RSV	24.0	54.0	55.6				22.0	125.2	25.8	4.4	120.8	15.0	41.2	2560
MSV(MLV)	1.2	7.2	9.6	0.07	15.2	15.2	1.5	14.8	1.4	0.07	19.0	2.0	1.9	3020
RSV + MSV(MLV)[e]							20.6[f]							5200

[a] The numbers represent picomoles of dNTP converted to acid-precipitable material after 60 min of incubation as described in Materials and Methods. Poly(A)·oligo(dT) was assayed by using ^{3}H-dTTP and all other templates by using ^{3}H-dCTP as radioactive substrate.

[b] Concentration of RNA was 2 μg per assay.

[c] Heat-dissociation of native viral RNAs was in standard buffer but containing only 0.01 M NaCl, at 100 C for 45 s in sealed ampules (8, 9).

[d] Oligo(dT) or oligo(dC) were used at a concentration of 1 μg per assay.

[e] The same number of units of RSV and MSV(MLV) enzyme were mixed.

[f] Concentration of RNA was 1 μg per assay.

zyme (Fig. 4A). This is in agreement with results described previously (9).

(ii). It had been shown that the template activity of 60 to 70S RSV RNA for RSV DNA polymerase is reduced 5 to 20-fold upon heat dissociation of the 60 to 70S RSV RNA complex into 30 to 40S and smaller RNA subunits (8, 9). This loss of template activity was attributed to the dissociation of 4S viral RNA primers from major 30 to 40S subunits of 60 to 70S RSV RNA (4). As shown in Table 4, heating also reduced about 20- to 30-fold the rather low template activities of Ki-MSV(MLV) RNA and of RSV RNA for Ki-DNA polymerase. It is further shown in Table 4 that the template activity of MSV(MLV) RNA for RSV DNA polymerase was reduced fivefold by heating. Thus it appears that native Ki-MSV(MLV) RNA contains heat-sensitive template-primer structures analogous to those found in 60 to 70S RSV RNA (4).

(iii). Oligo(dT) and oligo(dC) have been shown to enhance the template activity of 60 to 70S RSV RNA two- to threefold (9, Table 4) and that of heat-dissociated RSV RNA 20- to 30-fold for RSV DNA polymerase (9). It can be seen in Table 4 that this was similar for the response of the same templates to Ki-MSV(MLV) DNA polymerase. Further, it is shown in Table 4 that oligo(dT) enhances the template activity of 55S Ki-MSV(MLV) RNA sixfold for RSV DNA polymerase and 10-fold for MSV(MLV) DNA polymerase. The template activity of heated MSV(MLV) RNA was enhanced 30-fold for RSV DNA polymerase and over 250-fold for MSV(MLV) DNA polymerase by oligo(dT). Oligo(dC) did not affect the template activity of 55S MSV(MLV) RNA if assayed with either murine or avian DNA polymerase (Table 4). The template activity of heated MSV(MLV) RNA was increased threefold with RSV DNA polymerase and 30-fold with MSV(MLV) DNA polymerase by oligo(dC). We deduce from this that the relatively low efficiency of oligo(dC) in enhancing the template activity of MSV(MLV) RNA and the high efficiency of oligo(dC) in enhancing avian tumor virus RNA reflects distinctive properties of the two RNA. Presumably avian tumor virus RNA contains a G-rich sequence not present in Ki-MSV(MLV) RNA.

In addition, experiments were done in which the avian and the murine DNA polymerase were tested together with viral RNA templates and poly(A)·oligo(dT). The activities of both enzymes were additive in all these experiments (Table 4).

We conclude from all these experiments (Table 4 and Fig. 4) that per unit poly(A)·oligo(dT) dependent-DNA polymerase activity of the murine DNA polymerase is between 6 and 60 times less efficient than the avian DNA polymerase with the RNA or DNA templates tested (Table 4 and Fig. 4). It follows that the low efficiency of MSV(MLV) DNA polymerase in transcribing MSV(MLV) RNA is not due to a defect of the viral RNA but rather of the enzyme. However, it was observed consistently (Table 4) that the response of murine DNA polymerase to native or heat-dissociated viral RNA was enhanced more by oligo(dT) or oligo(dC) than the response of avian DNA polymerase to the same templates.

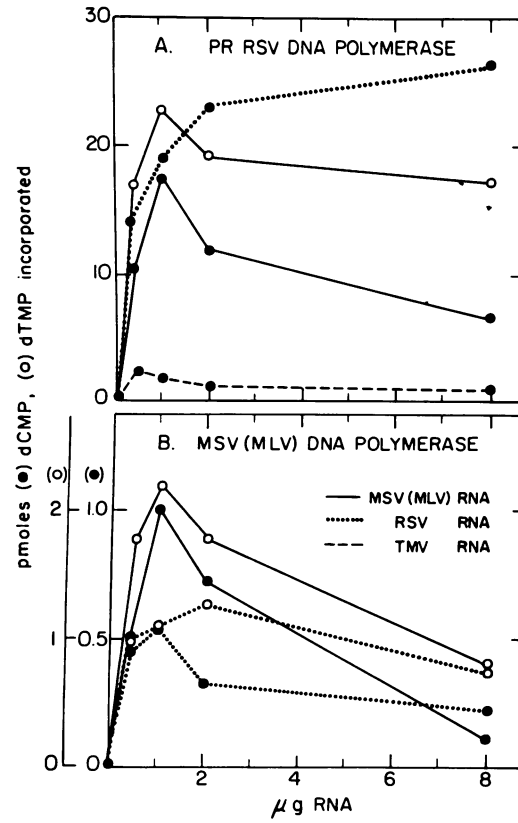

FIG. 4. *Dependence of DNA synthesis by PR RSV-B DNA polymerase (A) and Ki-MSV(MLV) DNA polymerase (B) on the concentration of viral RNA templates. Assays were for 120 min as described in Materials and Methods.*

DISCUSSION

The results described here suggest that the soluble DNA polymerase of murine RNA tumor viruses differs from that of avian tumor viruses in (i), its lack of detectable RNase H activity if

assayed with homopolymer and *fd* DNA-RNA hybrids as substrates and (ii), in its relatively low template activity for viral RNA and natural DNA.

Some of these observations agree with those made by others. It was found by J. P. Leis, J. Hurwitz, A. L. Schincariol, and W. K. Joklik, (Symp. on Biology of Tumor Viruses, 34th Annual Biol. Coll., Oregon State University, April 1973, personal communication) and by J. W. Abrell, and R. C. Gallo (J. Virol., in press) that the DNA polymerase of avian myeloblastosis virus (AMV) transcribes AMV RNA 50- to 100-fold better than the DNA polymerase from other mammalian tumor viruses and it was shown that Rauscher DNA polymerase is 200 times less active with activated salmon DNA than with poly(A)·oligo(dT) (27). Lack of RNase H activity was observed for the DNA polymerase of Rauscher leukemia virus (J. P. Leis et al., see above. 1973; personal communication) and for the DNA polymerase of other mammalian RNA tumor viruses (R. C. Gallo, 1973, personal communication). However our results disagree with an earlier report on Moloney MSV(MLV) DNA polymerase, which claims that this enzyme copurifies with RNase H (13).

It was postulated that DNA polymerase-associated RNase H (24) or a combination of DNA polymerase associated RNase H and cellular RNase H (19, 20) play a biological role in removing viral RNA from the putatitive RNA-DNA intermediates which are thought to occur in virus replication. The RNase H associated with the DNA polymerase of avian tumor viruses has been distinguished from cellular RNase H on the basis of enzymatic properties: the cellular RNase H is an endonuclease and the viral DNA polymerase-associated RNase H is a processive exonuclease (19, 20). The lack of RNase H activity in purified murine virus DNA polymerase may indicate that transcription of murine viral RNA to DNA, during virus replication, does not require a viral RNase H activity. However, it is conceivable that in murine RNA tumor virus replication a processive RNase H is also required. This may be a free enzyme either within the virion or it may be present in the host cell.

In an endogenous DNA polymerase reaction the RNA of Rous sarcoma virus was shown to be 80 to 100% transcribed to small fragments of DNA, although some sequences are transcribed much better than others (5, 28). Analogous results were reported for endogenous murine sarcoma-leukemia virus systems in which also 80 to 100% of the viral RNA was transcribed to DNA (11, 15, 26). From this it may be expected that purified murine DNA polymerase would also transcribe viral RNA completely and rather effectively to DNA. However, murine as well as avian tumor virus RNA are poor templates for purified murine virus DNA polymerase. By contrast, the same RNAs are good templates for avian viral DNA polymerase.

This has been corroborated for the endogenous DNA polymerase systems of PR RSV-B and Ki-MSV(MLV). Based on the same activities with poly(A)·oligo(dT), the avian system transcribed viral RNA 5 to 20 times better than the murine counterpart. For example, per 20 units of poly(A)·oligo(dt) primed polymerase activity, PR RSV-B (0.075 A_{260} units of virus) transcribed 57 pmol of viral RNA to DNA, while Ki-MSV(MLV) (0.05 A_{260} units) transcribed 12 pmol, and Mo-MLV (0.1 A_{260} units) transcribed 3.6 pmol of viral RNA to DNA. It is also possible that perhaps upon solubilization and purification of the murine enzyme a factor is lost that is essential for the transcription to DNA of viral RNA or denatured salmon DNA. Preliminary experiments in searching for such a factor by mixing soluble fractions of the disrupted virion have not been very successful so far. Some soluble fractions increased the template activity of viral RNA two- to fivefold (unpublished data).

It appears likely that DNA polymerase of avian tumor viruses with a molecular weight of 160,000 contains a protein factor enhancing transcription of natural RNA or DNA templates that is not present in the soluble DNA polymerase of murine viruses with a molecular weight of only 70,000. However, it has been claimed in one study, that even a subunit of avian DNA polymerase with a molecular weight of 65,000, termed α subunit, transcribes 60 to 70*S* viral RNA and natural DNA as effectively as the whole enzyme (14). Thus, further work is required to explain why avian tumor virus DNA polymerase can more effectively transcribe viral RNA and denatured salmon DNA than murine DNA polymerase.

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