JOURNAL OF VIROLOGY, Dec. 1974, p. 1494-1502

Vol. 14, No. 6
Printed in U.S.A.

Mechanistic Independence of Avian Myeloblastosis Virus DNA Polymerase and Ribonuclease H

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Received for publication 11 July 1974

Differential inhibition conditions were established for the DNA polymerase and RNase H activities of avian myeloblastosis virus (AMV) with ether-disrupted AMV and a purified enzyme preparation. The RNase H activity of ether-disrupted AMV with $(rA)_n \cdot (dT)_n$ and $(rA)_n \cdot (dT)_{11}$ as substrates was inhibited 80 to 100% by preincubation with NaF at a final reaction concentration of 27 to 30 mM. Under these conditions, the DNA polymerase activity was inhibited only 0 to 20%. Similar inhibitions were found with exogenous Rous sarcoma virus 35*S* and 70*S* RNA·DNA hybrid and ϕX174 DNA·RNA hybrid as substrates. Studies were also performed with a purified enzyme preparation, in which the two activities essentially co-purified. The RNase H activity was inhibited >80% by 150 mM KCl with three different hybrid substrates, whereas the DNA polymerase activity was uninhibited. The DNA polymerase was completely inactivated by heat denaturation at 41 C or by omission of the deoxytriphosphates from the reaction mixture; the RNase H remained active. These differential inhibition conditions were used to compare the size of the DNA product synthesized with and without simultaneous RNase H action and to examine the effect of inhibition of the DNA polymerase on the size of the RNase H products. The size of the products of one activity was not affected by inhibition of the other activity. These results suggest that the AMV DNA polymerase and RNase H are not coupled mechanistically.

RNase H, which specifically degrades the RNA strand of a DNA·RNA hybrid, is one of the numerous nucleic acid metabolizing activities associated with RNA tumor viruses. Several recent studies (1, 2, 9, 10, 13, 14, 15, 22, 28) demonstrated the co-purification of the avian myeloblastosis virus (AMV) RNase H and DNA polymerase activities, and models were proposed which described a role for RNase H in the replication cycle of these viruses (5, 13, 14, 22). In addition, there was a precedent for exonucleases as integral parts of DNA polymerases: both the *Escherichia coli* (4) and *Micrococcus luteus* (18, 19) DNA polymerases are also 5'-specific exonucleases which are specific for double-stranded nucleic acids to give oligonucleotides as products. Hence, the notion of coupled enzyme activities was readily accepted.

In an effort to understand the process of DNA synthesis in RNA tumor virus systems, we wished to determine if the mechanisms of the AMV DNA polymerase and RNase H activities were obligately coupled. Since attempts at physical separation of the two activities in AMV had been unsuccessful to date, our experimental approach was to establish conditions under which one of the activities was preferentially inhibited, leaving the other activity essentially unaltered. Inhibition studies were performed with ether-disrupted AMV and with a purified enzyme preparation using four template·primer/substrate mixtures. The conditions which resulted in preferential inhibition of one activity were used to study the size of the products of the other activity. The results indicate that there is no apparent coupling between the mechanisms of the AMV DNA polymerase and RNase H.

MATERIALS AND METHODS

Nucleic acids. ϕX174 DNA·[^{3}H]RNA was prepared by transcribing ϕX174 DNA with *E. coli* RNA polymerase as described previously (R. M. Wartell, J. E. Larson, and R. D. Wells, J. Biol. Chem., in press) and was purified further by hydroxyapatite chromatography. [^{32}P]Rous sarcoma virus (RSV) 70*S* and 35 *S* RNA (gift of A. M. Q. King) was isolated as described (6; unpublished work). This RNA was used instead of labeled AMV RNA due to the ease of purification of a high-specific-activity, relatively non-fragmented RNA from RSV. However, some studies were performed with ^{3}H-labeled 70*S* AMV RNA, and

similar results were obtained with both RNAs. [^{3}H](rA)$_n$ was purchased from Schwarz/Mann, Inc. or was prepared (26) with polynucleotide phosphorylase (Miles) to obtain a higher specific activity (90 mCi/mmol). Nonradioactive (rA)$_n$ and (rU)$_n$ were gifts of J. E. Larson. All synthetic RNAs were purified as described previously (29). (dT)$_n$ was prepared with the calf thymus terminal addition enzyme or with the *M. luteus* DNA polymerase (11, 18) followed by strand separation (3). The preparations had molecular weights (30) ranging from 1×10^5 to 13×10^5. (dT)$_{10}$ and (dT)$_{11}$ were prepared and characterized by R. W. Sweet and T. Tamblyn (unpublished work). (rA)$_8$ was a gift from U. RajBhandary. Unlabeled and labeled triphosphates were purchased and checked for purity as previously described in systems A-C (6, 29, 31).

Enzyme preparations. Avian myeloblastosis virus (in plasma) from J. W. Beard was stored at −20 C. The virus was purified (24) and disrupted with ether essentially as described previously (6-8).

The purified AMV DNA polymerase-RNase H preparation was obtained using the following steps: ether-disruption of purified virus, sucrose gradient centrifugation, and DEAE-Sephadex column chromatography. The enzyme activity was eluted from the DEAE-Sephadex column with 0.01 M potassium phosphate buffer (pH 6.9), 25% ethylene glycol, 5 mM dithiothreitol, 1% Nonidet P-40, and 0.3 M NaCl. The column fractions were assayed for RNase H and DNA polymerase activities as described below using (rA)$_n$·(dT)$_n$ as substrate or template·primer. The ratio of the two activities varied as much as fivefold across the column peak; both activities were assayed in the linear range of enzyme concentration. In some preparations, tailing was observed with the RNase H activity, whereas the DNA polymerase consistently eluted as a sharp peak. The purified enzyme preparation rendered 590 nmol of [^{3}H](rA)$_n$ acid soluble per mg of protein in 90 min in a standard [^{3}H](rA)$_n$·(dT)$_n$ assay and incorporated 615 nmol of dTMP per mg of protein in 60 min in the standard (rA)$_n$·(dT)$_n$ assay. Throughout the purification, the ratio of the two activities (total units of DNA polymerase to total units of RNase H) at the various stages varied no more than twofold; however, a greater variance in this ratio was found across the DEAE-Sephadex column.

The enzyme preparation was not homogeneous as judged by SDS-polyacrylamide gel electrophoresis. It contained no detectable phosphatase or DNase activity, but it did contain nucleoside diphosphokinase activity as reported previously (17). The purification procedure was originally designed by P. F. Schendel (this laboratory); details of the procedure are available on request to the authors.

DNA polymerase assay. The DNA polymerase activity was assayed with the synthetic template·primers, (rA)$_n$·(dT)$_n$ and (rA)$_n$·(dT)$_{11}$, and also with the natural templates, ϕX174 DNA·RNA hybrid, AMV 70*S* RNA, and RSV 70*S* and 35*S* RNA. Unless stated otherwise, the standard 0.1-ml reaction contained: Tris-hydrochloride buffer (pH 8.1), 50 mM; $MgCl_2$, 10 mM; NaCl or KCl, 30 to 60 mM; dithiothreitol, 20 mM; rATP, 0.5 mM; (rA)$_n$ and (dT)$_n$ or (dT)$_{11}$, 60 μM each; dTTP, 108 μM; [^{3}H]dTTP or α[^{32}P]dTTP, $\geq 1 \times 10^6$ counts per min per reaction; and enzyme as indicated. When necessary, the enzyme was diluted with dilution buffer (0.05 M Tris-hydrochloride (pH 8.1), 5.0 mM $MgCl_2$, 2.0 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin). The polynucleotide template·primer components were mixed and annealed at 65 C for 10 min and then slowly cooled. When natural templates were utilized instead of the synthetic polymers, the same conditions were used except that all four dNTPs were added, three unlabeled at concentrations of 90 μM each and the labeled dNTP at a concentration of 10 μM. The concentrations of the AMV 70*S* RNA and RSV 70*S* and 35*S* RNA were not known; ϕX174 hybrid was used at a final reaction concentration of 40 μM. Control reactions (no enzyme) had backgrounds of 10 to 20 pmol/ml. The reactions were incubated at 41 C for the length of time indicated. The incorporation of radioactive dNMP into acid-insoluble material was monitored essentially as described previously (11, 23).

RNase H assay. The RNase H activity was assayed with the following hybrid substrates: [^{3}H](rA)$_n$·(dT)$_n$, [^{3}H](rA)$_n$·(dT)$_{11}$, ϕX174 DNA·[^{3}H]RNA, [^{32}P]35*S* or 70*S* RSV RNA, and [^{3}H]70*S* AMV RNA. The RSV and AMV RNAs were used with the DNA transcribed from them by AMV DNA polymerase to provide the hybrid structure for RNase H. The (rA)·(dT) hybrid components were mixed in an equimolar ratio, heated to 65 C for 10 min, and then slowly cooled.

The components and conditions of the standard RNase H reactions were the same as for the DNA polymerase reactions, except that the labeled dNTPs were omitted. At specific time intervals, portions were withdrawn and acid-soluble radioactivity was determined as previously described (16, 18). The control assays for single-stranded and double-stranded RNases were the same as the standard assay, except that [^{3}H](rA)$_n$ or [^{3}H](rA)$_n$·(rU)$_n$ were used as substrates.

Size analysis of the DNA polymerase products. The DNA polymerase reactions were prepared as described above with conditions varied to inhibit the RNase H activity as indicated. The reactions were stopped by adding EDTA (pH 7.5) to a final concentration of 50 mM on ice. Potassium hydroxide was added (0.3 M final concentration) to hydrolyze the RNA template. After hydrolysis at 37 C for 16 h, the alkali was neutralized by titrating with HCl or by adding Dowex-50 in the acid form. The product then was dialyzed essentially as described (31) to remove the dNTPs and salt, or the product was desalted by column chromatography on Sephadex G-50 columns (2.4 cm^3 column volume, 0.75 cm diameter). The method of neutralization and salt removal had no influence on the size of the product although the recoveries varied from 50 to 90%. The purified products were layered onto 5 to 20% sucrose (in 0.01 M Tris-hydrochloride, pH 8.1; 0.1 M NaCl; 10^{-3} M EDTA) gradients and were centrifuged at 40,000 rpm for 4 h in an SW50.1 rotor. The gradients were dripped into 200-μliter fractions. The acid-insoluble radioactivity in 20- to 100-μliter volumes and the density

across the gradients were determined. The recovery from the sucrose gradients was 80 to 100%.

Other methods. Descending paper chromatography on Whatman #1 paper was performed in the following systems: (A) isobutyric acid-concentrated ammonium hydroxide-H_2O (66:1:33); (B) ethanol-1.0 M ammonium acetate buffer (pH 7.5) (7:3); (C) 0.1 M sodium phosphate (pH 6.8)-ammonium sulfate-*n*-propanol (100:60:2, vol/wt/vol). Radioactivity was detected by liquid scintillation counting on 1-cm strips of the chromatograms. Other details were described previously (7, 8, 29).

RESULTS

Characteristics of the enzyme preparations. Studies were performed with both ether-disrupted AMV and a purified DNA polymerase-RNase H preparation using several template·primers/substrates. Before differential inhibition attempts were made, studies were performed to establish the linear range of enzyme concentration for the DNA polymerase and RNase H activities in order to determine their relative amounts.

Ether-disrupted AMV. The RNase H activity, measured by the degradation of [^{3}H](rA)$_n$ hybridized with (dT)$_n$, was essentially linear over the range of 0 to 5.0 μliters of disrupted AMV in a 100-μliter reaction; 8 nmol of [^{3}H](rA)$_n$ per ml was made acid soluble in 60 min by 5 μliters of disrupted AMV. The reaction kinetics were linear for at least 90 min of incubation at 41 C with an enzyme concentration of 20 μliters or less in a 100-μliter reaction (data not shown). The reaction using [^{3}H](rA)$_n$·(dT)$_{11}$ was also linear for at least 90 min of incubation at 41 C with 20 μliters of AMV per 100-μliter reaction; however, the extent of degradation in 1 h was approximately 1.3-fold greater than observed with [^{3}H](rA)$_n$·(dT)$_n$.

The DNA polymerase activity, measured by the incorporation of dTMP into acid-insoluble material with (rA)$_n$·(dT)$_n$ as template·primer, was linear over the concentration range of 0 to 5.0 μliters of enzyme in a 100-μliter reaction; 18 nmol of dTMP per ml was incorporated in 60 min by 5 μliters of disrupted AMV. The DNA polymerase reaction with (rA)$_n$·(dT)$_n$ remained linear for only the first 30 min of incubation at 41 C with 5.0 μliters or more of disrupted AMV in a 100-μliter reaction. With less than 5.0 μliters of enzyme in the reaction, synthesis proceeded linearly for at least 90 min. Similar results were found with (rA)$_n$·(dT)$_{11}$ (data not shown). The amount of DNA synthesis in 1 h with (rA)$_n$·(dT)$_{11}$ was approximately threefold greater than with (rA)$_n$·(dT)$_n$. When (rA)$_n$·(dT)$_n$ was considered, the ratio of the amount of dTMP incorporated to the amount of [^{3}H](rA)$_n$ made acid-soluble with 2 μliters of enzyme after 60 min was approximately 3:1. Studies in this and other laboratories (1, 2, 5–7, 9, 13, 22) have established optimum reaction conditions for the disrupted AMV DNA polymerase and RNase H activities.

The presence of ribonucleases (12, 25) other than RNase H was studied by providing (rA)$_n$ or (rA)$_n$·(rU)$_n$ as substrate. The amount of activity observed with these substrates was only 3.0 and 1.4%, respectively, of that found using the hybrid substrate. Thus, RNase H was the predominant RNase under these assay conditions.

Purified DNA polymerase-RNase H preparation. The purified enzyme preparation, in which the DNA polymerase and RNase H activities essentially co-purified, was studied to determine maximum reaction conditions and to quantitate the relative amounts of the two activities. The influence of the following variables on the enzyme activities was determined: (dT)$_n$ concentration from 0 to 160 μM or the (dT)$_{11}$ concentration from 10 to 200 μM with the (rA)$_n$ concentration constant at 40 μM; (rA)$_n$·(dT)$_n$ concentration from 0 to 70 μM; deoxytriphosphate concentration from 0 to 0.18 mM; the divalent metal ion ($MgCl_2$, $MnCl_2$, or $MgCl_2$ + $MnCl_2$); and $MgCl_2$ concentration from 0 to 30 mM. The optimum reaction conditions are described in Materials and Methods. Detailed results of these studies are not described herein but are available on request from the authors.

Experiments were then performed to determine the linear range of enzyme concentration for the purified activities. The ratio of the amount of dTMP incorporated to the amount of [^{3}H](rA)$_n$ made acid soluble in 2 h with (rA)$_n$·(dT)$_n$ was approximately 1:1. Since the RNase H activity was more stable during storage (unpublished data; 22), this ratio changed after storage of the enzyme preparation at 5 C.

Inhibition studies. To study the association and the possible coupling of the two activities, it seemed appropriate to find methods that would preferentially inhibit one of the activities, leaving the other activity essentially uninhibited. Several reaction requirements and inhibitors were studied.

NaF inhibition. Results obtained previously in this lab (5) indicated that an appropriate concentration of NaF would inhibit the RNase H and not the DNA polymerase activity in ether-disrupted AMV. The effect of 0 to 75 mM NaF (final reaction concentration) on the DNA polymerase and RNase H reactions with (rA)$_n$·(dT)$_n$ is shown in Fig. 1. At 24 to 30 mM

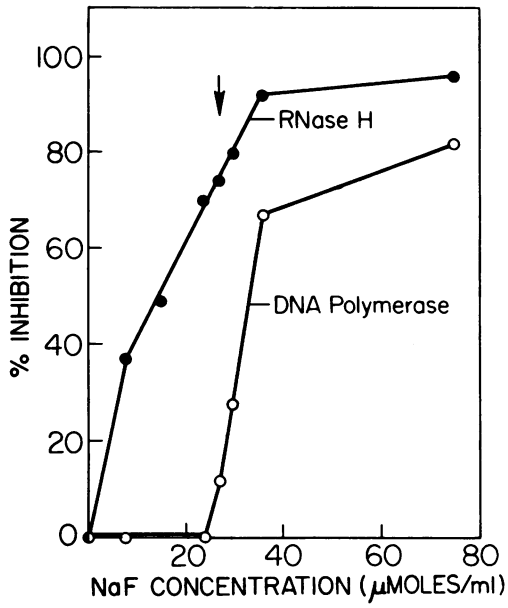

FIG. 1. *Sodium fluoride inhibition of DNA polymerase and RNase H in ether-disrupted AMV. The DNA polymerase and RNase H activities were assayed as described in Materials and Methods using* $(rA)_n \cdot (dT)_n$ *and* $[^3H](rA)_n \cdot (dT)_n$, *respectively. A 20-μliter volume of disrupted AMV was used in each 100-μliter reaction after preincubation with an amount of NaF necessary to provide the indicated final concentrations after addition to the reaction mixtures. The preincubations were for 30 min at 0 C. The KCl concentration of the reactions was decreased as the NaF concentration was increased to maintain a constant salt concentration. The arrow at 27 mM indicates the inhibitor concentration used for further studies.*

NaF, the RNase H was inhibited greater than 70%, whereas the DNA polymerase was inhibited less than 25%. Titration experiments also were performed using $(rA)_n \cdot (dT)_{11}$, and the inhibition patterns were similar to those observed with $(rA)_n \cdot (dT)_n$ (data not shown). The RNase H reaction kinetics using $(rA)_n \cdot (dT)_{11}$, with and without 27 mM NaF, were linear; the reaction was inhibited 87% in 90 min by 27 mM NaF. Alternatively, kinetic analysis showed that the DNA polymerase reaction was inhibited only 15% in 30 min and 27% in 90 min with 27 mM NaF (results not shown).

The NaF inhibition effect on the RNase H of ether-disrupted AMV was confirmed with $[^{32}P]$70*S* and 35*S* RSV RNA and φX174 DNA · $[^3H]$RNA as substrates. In all cases, at least 70% inhibition (average of 81% for four test cases) was observed after preincubation of the disrupted AMV with 30 mM NaF (final reaction concentration) for 30 min at 0 C. Sucrose gradient analysis and chromatographic analysis of the $[^{32}P]$RSV RNA after a 4-h reaction with disrupted AMV also showed greater than 80% inhibition of the RNase H under these NaF preincubation conditions. Therefore, preincubation of ether-disrupted virus with NaF provided a useful tool for studying the DNA polymerase products synthesized in the absence of RNase H.

Studies also were performed with the purified enzyme preparation to determine the NaF inhibition patterns. The ability of NaF to inhibit the RNase H was approximately the same as in the disrupted virus system. However, the purified DNA polymerase activity was appreciably more sensitive (96% inhibition at 27 mM) than the disrupted AMV DNA polymerase. Similar inhibition patterns were found using both $(rA)_n \cdot (dT)_n$ and $(rA)_n \cdot (dT)_{11}$ in the reactions. This difference in the two systems was at least partially due to the presence of Nonidet P-40 in the purified enzyme fraction. When Nonidet P-40 (final reaction concentration of 2.0%) was added to the ether-disrupted AMV reactions, the DNA polymerase activity became more sensitive to NaF (80% inhibition at 27 mM).

Inhibition with KCl. Previous work suggested that high salt concentrations were inhibitory to the purified AMV RNase H activity (1, 13, 15). Titration studies on the effect of KCl concentration on the two activities showed that the RNase H activity was more sensitive than the DNA polymerase with the purified enzyme (Fig. 2). At 150 mM KCl, the DNA polymerase remained uninhibited (less than 10%), whereas the RNase H was inhibited greater than 80%. The kinetics of the two reactions at 30 and 150 mM KCl using $(rA)_n \cdot (dT)_n$ were studied over a 2-h period. The RNase H activity was inhibited at least 80% throughout the time course by 150 mM KCl; the DNA polymerase was not inhibited by the same treatment. In fact, a slight stimulation (up to 125%) of the DNA polymerase activity by 150 mM KCl frequently was observed. Therefore, the use of 150 mM KCl in the reactions provided a method for studying the DNA polymerase reaction in the absence of the RNase H activity.

The inhibition of RNase H by high salt also was observed with φX174 DNA · $[^3H]$RNA as the substrate. The RNase H reaction was inhibited at least 60% by 150 mM KCl and 91% by 250 mM KCl. Due to the low amount of DNA synthesis with φX174 DNA · RNA as template, it was not possible to determine the effect of KCl concentration on the DNA polymerase reaction.

In contrast to the results with the purified

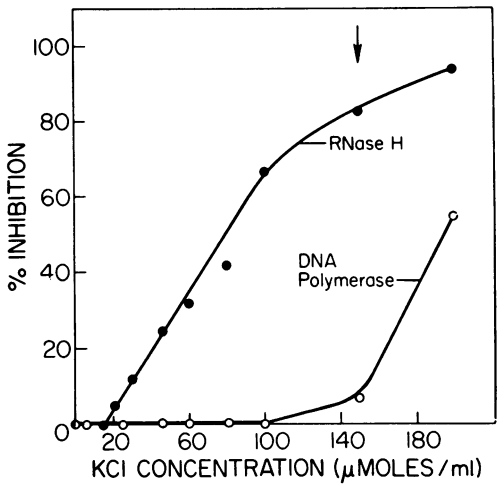

FIG. 2. *Potassium chloride inhibition of the purified AMV DNA polymerase and RNase H activities. The DNA polymerase and RNase H activities were assayed using* $(rA)_n \cdot (dT)_n$ *and* $[^3H](rA)_n \cdot (dT)_n$*, respectively. A 15-μliter volume of enzyme was used in each 100-μliter reaction; the assays were performed as described in Materials and Methods, except that the KCl concentration was varied from 0 to 200 mM as indicated. The arrow at 150 mM KCl indicates the concentration used in further studies.*

enzyme, the RNase H activity of the disrupted AMV using $(rA)_n \cdot (dT)_n$ was less sensitive to salt inhibition; only 25% inhibition was found at 150 mM KCl. As seen for the purified enzyme, the DNA polymerase was not inhibited; in fact, the DNA polymerase was slightly stimulated (up to 128%) by the higher KCl concentrations. In an effort to understand the difference between the inhibitions found in the two systems, Nonidet P-40 (2.0% final concentration) was added to the ether-disrupted virus reactions. No change was found for the DNA polymerase reactions; however, the RNase H was more sensitive to KCl inhibition ($\geq$40% inhibition at 150 mM KCl). Thus, it is apparent that subtle differences in reaction conditions influence the observed inhibition patterns.

Heat inactivation studies. Figure 3 shows that the purified DNA polymerase using $(rA)_n \cdot (dT)_n$ was inactivated greater than 80% by preincubation at 41 C for 15 min, whereas the RNase H remained greater than 99% active. After preincubation for 15 min at 65 C, the RNase H still was inactivated only 42%. When heat inactivation studies were performed with $(rA)_n \cdot (dT)_{11}$, the DNA polymerase was inactivated 50% after 5 min at 41 C, 80% after 15 min at 41 C, and 100% after 5 min at 50 C.

Therefore, heat denaturation of the purified enzyme provided a method for studying the RNase H degradation in the absence of the DNA polymerase activity. Differential heat denaturation of the DNA polymerase and RNase H in a purified AMV enzyme preparation had been suggested previously (14, 15).

Attempts were made to preferentially inactivate the DNA polymerase activity in disrupted AMV by heat treatment. The RNase H was substantially more sensitive to the treatment in disrupted AMV (82% inactivation by preincubation for 15 min at 41 C) than in the purified system; the DNA polymerase inactivation was not markedly different. Therefore, this technique was not applicable to the disrupted virus system as a tool for preferential inhibition of the DNA polymerase activity.

Effect of dNTPs on RNase H activity. Previous reports indicated that dNTPs were not required for the AMV RNase H reaction when using $[^3H](rA)_n \cdot (dT)_n$ or natural hybrids (1, 2, 9, 10, 14, 15, 22, 28). Results of studies with [^{3}H]poly(rA)·oligo(dT) as substrate indicated that the omission of dTTP was inhibitory (1, 2, 14, 15). The effect of dNTPs on the RNase H activity of the purified enzyme was examined using $[^3H](rA)_n \cdot (dT)_n$, $[^3H](rA)_n \cdot (dT)_{11}$, and

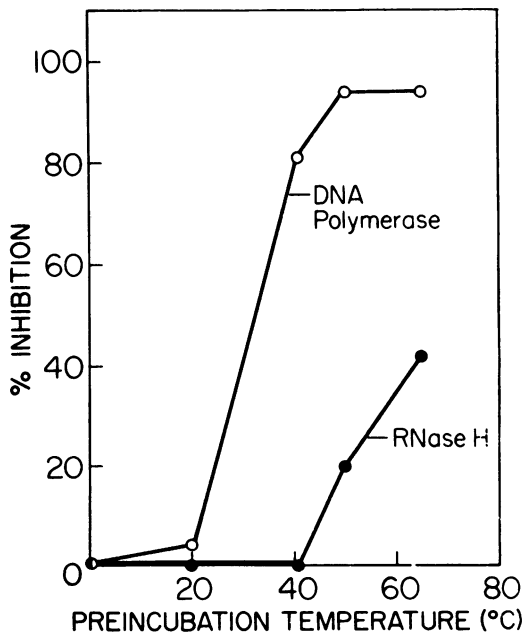

FIG. 3. *Effect of heat treatment on the purified AMV DNA polymerase and RNase H. The DNA polymerase and RNase H activities were assayed using* $(rA)_n \cdot (dT)_n$ *and* $[^3H](rA)_n \cdot (dT)_n$*, respectively. The enzyme (20 μliters at* $\leq$*0.04 mg/ml in 0.25 M Tris-hydrochloride, pH 8.1) was preincubated for 15 min at the indicated temperatures. The assays then were performed as described in Materials and Methods.*

ϕX174 DNA·[^{3}H]RNA as substrates. Three (rA)-to-(dT) ratios (5:1, 2:1, and 1:1) were studied with both $(rA)_n \cdot (dT)_n$ and $(rA)_n \cdot (dT)_{11}$. If the dNTPs were omitted, or a triphosphate was included which could not be incorporated [i.e., dCTP in an (rA)·(dT) reaction], less than 20% inhibition was observed in all cases. Therefore, the omission of dNTPs provided a second method for inactivating the DNA polymerase and retaining RNase H activity with the purified enzyme.

Although numerous techniques were studied, no completely satisfactory method was found for preferential inhibition of the DNA polymerase in the disrupted virus preparation. Omission of dNTPs should, in principle, inhibit the DNA polymerase activity. However, prior studies showed the presence of nucleotides and nucleotide kinases in disrupted virus preparations (20, 21). Thus it was not possible to be certain of the absence of substrates for the DNA polymerase. A reaction was performed using only the labeled deoxytriphosphate (0.25 μM [^{3}H]dTTP; no unlabeled dNTP was added). If the assumption was made that the only substrate used by the enzyme was that added as labeled dTTP, the amount of DNA synthesized was only 10% that synthesized under normal reaction conditions.

To test the effect of the apparent absence of a DNA polymerase reaction on the RNase H activity, studies were performed in the absence of added dNTPs. When either [^{3}H]$(rA)_n \cdot (dT)_n$ or ϕX174 DNA·[^{3}H]RNA was used as the substrate, less than 5% inhibition was observed when the dNTPs were omitted. However, when the dTTP was omitted with [^{3}H]$(rA)_n \cdot (dT)_{11}$ as substrate, inhibition of the RNase H was observed. At a 1:1 (rA)-to-(dT) ratio, 50% inhibition resulted; 80% inhibition resulted at a 2:1 ratio; and 90% inhibition was observed at a 5:1 ratio. Therefore, when [^{3}H]$(rA)_n \cdot (dT)_n$ and ϕX174 DNA·[^{3}H]RNA hybrid substrates were used, there was no dNTP requirement for the RNase H activity. However, when [^{3}H]$(rA)_n \cdot (dT)_{11}$ was used as the substrate, omission of dTTP from the reaction resulted in inhibition of the disrupted AMV RNase H.

Other inhibition studies. Other inhibitors were tested including 1,10-phenanthroline, iodoacetate, $HgCl_2$, and *N*-ethylmaleimide. In no case were the differential inhibitions as pronounced as found above; however, some preferential inhibition of the purified DNA polymerase was found with 1,10-phenanthroline, iodoacetate, and *N*-ethylmaleimide.

Effect of RNase H inhibition on the size of the DNA polymerase products. The NaF and KCl inhibition results discussed above provided tools for studying the possible role of RNase H in DNA synthesis by the AMV DNA polymerase. Size analysis was performed on the DNA polymerase products to determine if the size of the products was different when the RNase H activity was inhibited.

The DNA polymerase reactions were performed under standard reaction conditions using $(rA)_n \cdot (dT)_{11}$ and ether-disrupted AMV, with and without NaF preincubation. The products from a 90-min reaction were alkali treated to hydrolyze the RNA template and centrifuged on 5 to 20% sucrose gradients as described in Materials and Methods. Figure 4 shows that the DNA polymerase synthesized a 6*S* product in both reactions. Studies using ether-disrupted AMV and $(rA)_n \cdot (dT)_n$, with and without NaF preincubation, gave similar results.

The size of the DNA polymerase product synthesized by the partially purified enzyme with $(rA)_n \cdot (dT)_n$ also was examined. The reactions were performed at both 30 and 150 mM KCl as described in Fig. 2. The products synthesized in 90 min were alkali treated and analyzed on sucrose gradients as described in Materials and Methods. The product synthesized in the presence of 150 mM KCl was 6*S*, whereas that formed in 30 mM KCl was 9*S* (results not shown). However, this size difference was probably a result of the high salt concentration and not a direct result of the inhibition of RNase H. When ether-disrupted virus was used with $(rA)_n \cdot (dT)_n$, the product was 12 to 13*S* (with and without NaF preincubation). High salt (150 mM KCl) had very little effect on the RNase H activity of ether-disrupted AMV. However, the DNA product synthesized by the disrupted virus system with 150 mM KCl was 3*S* units smaller than that synthesized with 30 mM KCl.

Therefore, when either $(rA)_n \cdot (dT)_n$ or $(rA)_n \cdot (dT)_{11}$ was used as template·primer, inhibition of the RNase H did not affect the size of the product synthesized by the DNA polymerase.

The size of the DNA polymerase product synthesized with ether-disrupted AMV in 5 h using 70*S* RSV RNA as the template also was examined. The reactions were performed with and without preincubation with NaF. The products were alkali treated and centrifuged on 5 to 20% sucrose gradients as described in Materials and Methods. Figure 5 shows that the DNA polymerase synthesized a 6 to 7*S* product in both reactions. The same result was observed when the endogenous disrupted AMV DNA polymerase reaction was studied. Therefore, as observed with the (rA)·(dT) reactions, inhibi-

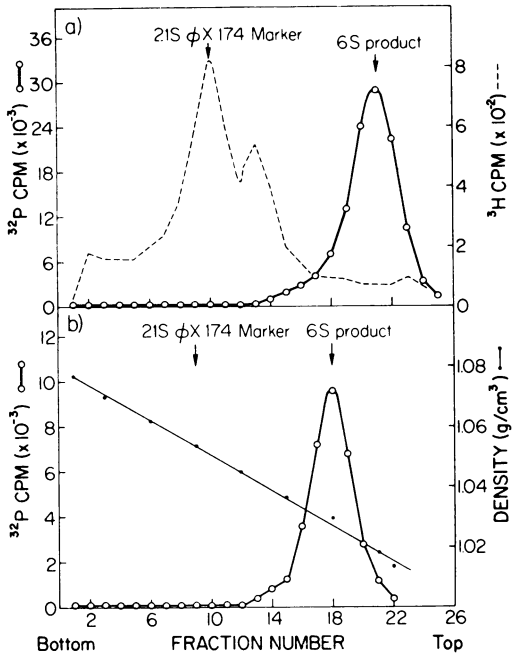

FIG. 4. *Effect of NaF inhibition of ether-disrupted AMV RNase H on the size of the DNA polymerase products. The reactions were performed as described in Materials and Methods using* $(rA)_n \cdot (dT)_{11}$ *as template·primer with 20 μliters of disrupted AMV in each 100-μliter reaction. The products synthesized in 90 min were purified as described in Materials and Methods. A 21S [*^{3}*H]φX174 RFI DNA marker (gift of J. B. Dodgson) was used in each gradient. This DNA was purposely nicked to give some RFII DNA (16 to 17S) in order to have a second molecular weight standard. The small amount of fast-sedimenting DNA is form IV. The density across the gradients was determined picnometrically. A 25-μliter volume of each 200-μliter fraction was used to determine the acid-precipitable radioactivity profile. (a) No preincubation with NaF; (b) preincubation with 27 mM NaF (final reaction concentration) for 30 min at 0 C. The KCl concentration was decreased in the reaction with NaF in order to maintain a constant salt concentration.*

tion of the RNase H in the disrupted virus did not appear to affect the size of the product synthesized using viral RNA as the template.

Effect of inhibition of the DNA polymerase on the size of the RNase H products. The preferential inhibition of the DNA polymerase activity was discussed above. Size analysis was performed on the $[^{3}H](rA)_n \cdot (dT)_n$ degradation products formed by RNase H to determine if products of a different size resulted when the DNA polymerase activity was inhibited.

The RNase H reactions using the purified enzyme with $[^{3}H](rA)_n \cdot (dT)_n$ were performed under three different conditions: (i) standard reaction conditions, (ii) standard conditions except that dTTP was omitted, and (iii) standard conditions except that the DNA polymerase was heat inactivated before addition of the enzyme to the reaction mixture. In all three cases, approximately 1% of the total radioactivity was rendered acid-soluble (Fig. 6), and greater than 70% of the acid-soluble products migrated in a relatively sharp peak just ahead of the $(rA)_8$ marker.

The RNase H reactions using disrupted AMV were performed under standard reaction conditions, except that in one case the dTTP was omitted to inhibit the DNA polymerase. Approximately 20% of the total radioactivity was rendered acid soluble; 50 to 60% of the acid-

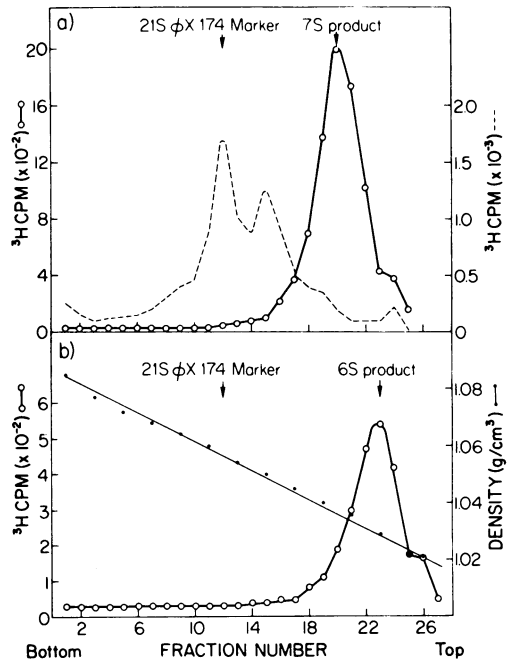

FIG. 5. *Effect of NaF inhibition of RNase H on the size of the DNA polymerase product using 70S RSV RNA as template with ether-disrupted AMV. The reactions were performed essentially as described in Fig. 4, except that 70S RSV RNA was used as the template with* $(dT)_{10}$ *(29 μM) as exogenous primer. All four dNTPs were included as substrates as described in Materials and Methods. The products synthesized in 5 h were purified as described in Materials and Methods. A gradient was also run which contained the 21S [*^{3}*H]φX174 RFI DNA marker. The density across the gradients was determined, and 50 μliters of each 200-μliter fraction was used to determine the acid-precipitable radioactivity profile. (a) No preincubation with NaF; (b) preincubation with 30 mM NaF as in Fig. 4.*

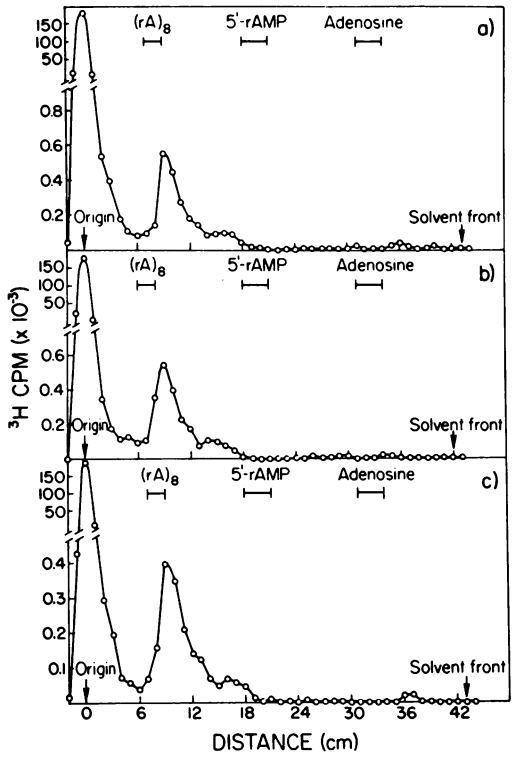

FIG. 6. *Effect of inhibition of the DNA polymerase activity on the RNase H products formed with the purified DNA polymerase-RNase H preparation. The reactions were performed with* [^{3}H]$(rA)_n \cdot (dT)_n$ *as described in Materials and Methods, except that in one case the dTTP was omitted and in another the enzyme was heat treated at 41 C for 15 min to inactivate the DNA polymerase activity. The 3-h reaction mixtures and the markers were spotted, chromatographed in system A, and analyzed as described in Materials and Methods. Three markers were used:* $(rA)_8$, *adenosine, and 5'-rAMP. The chromatogram was cut into 1-cm strips and the radioactivity was determined by liquid scintillation counting. (a) dTTP included in the reaction; (b) dTTP omitted from the reaction; (c) DNA polymerase activity heat inactivated.*

soluble product migrated in a relatively sharp peak just ahead of the $(rA)_8$ marker (results not shown). The remainder of the acid-soluble product was predominantly smaller oligomers. When [^{32}P]70S RSV RNA was degraded by the disrupted AMV RNase H, the products were the same size as those described above for the [^{3}H]$(rA)_n \cdot (dT)_n$ reaction.

Therefore, the products of the RNase H reaction were oligonucleotides (two to eight nucleotides in length), and inactivation of the DNA polymerase did not affect the size of the RNase H products.

DISCUSSION

Different reaction requirements and different sensitivities to several inhibitors of DNA polymerase and RNase H were observed with both ether-disrupted AMV and the purified enzyme preparation. When size analyses were performed on the DNA polymerase reaction products, no differences were observed when the RNase H activity was inhibited. In addition, inhibition of the DNA polymerase activity did not affect the size of the RNase H products. These results, in conjunction with the slight separation of the two activities during purification, demonstrate a lack of obligate coupling of the two reactions and indicate that the two activities have different active sites. The two active sites may be on the same or on different protein molecules.

Other recent studies are consistent with the notion of the non-necessity of RNase H for in vitro reverse transcriptase activity. Wang and Duesberg (27) reported that Kirsten murine sarcoma-leukemia virus DNA polymerase lacked detectable RNase H activity. In addition, R. C. Gallo et al. (personal communication) recently purified and characterized a reverse transcriptase from human leukemic cells and found little or no RNase H associated with the DNA polymerase; these authors also found a similar result for murine RNA tumor virus reverse transcriptases (32).

Although our results show that the two activities are not closely coordinated, as found for other DNA polymerase-exonucleases (4, 18, 19), it is still possible that RNase H has a vital role in provirus synthesis. Inhibition of RNase H had no influence on the size of the products formed by the DNA polymerase under the reaction conditions employed. However, it is conceivable that the nuclease could have a role in other aspects of DNA synthesis. For example, the RNase H could influence the secondary structure of the DNA product, the rate of release of the DNA product from the RNA template (primer?), or the extent of transcription of the template. The differential inhibition conditions described in this paper provide a tool for further study of the possible roles of AMV RNase H in DNA synthesis by the AMV DNA polymerase.

ACKNOWLEDGMENTS

We thank P. F. Schendel and R. M. Flügel for assistance and advice and J. and D. Beard for generously providing AMV.

This work was supported by Public Health Service funds from the National Cancer Institute (CM-12275) and the National Science Foundation (GB-30528X). One of us (L. C.

B.) was supported, in part, by a Public Health Service Training Grant to the Department of Biochemistry from the National Institute of General Medical Science (GM 00236-14).

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