ELIMINATION OF RESIDUAL RNase ACTIVITY FROM PURIFIED PREPARATIONS OF RNA-DIRECTED DNA POLYMERASE

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SUMMARY

Preparations of RNA-directed DNA polymerase purified from RNA tumor viruses by standard methods generally contain trace amounts of single-stranded RNA endonucleolytic activity detectable only by relatively sensitive methods. This contaminating RNase activity has been found to be completely inhibited when RNA-directed DNA polymerase reactions are carried out in the presence of low concentrations of bentonite. Under these conditions, only minimal inhibition of the DNA polymerase and RNase H activities of the RNA-directed DNA polymerase was observed.

INTRODUCTION

The RNA-directed DNA polymerase associated with RNA tumor viruses has been purified by a wide variety of procedures, most of which have consisted of various combinations of salt fractionation, ion-exchange chromatography, gel filtration, velocity centrifugation and affinity chromatography (1-7). The purity of oncornavirus DNA polymerase preparations has been assessed by determinations of specific activity, analysis of protein bands by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and assays for the presence of nuclease activities (other than RNase H) (8, 9). We have been interested in the in vitro transcription of the avian oncornavirus 70S RNA genome into DNA in an effort to elucidate the reaction mechanisms of reverse transcription. A necessary prerequisite for our studies is highly purified preparations of RNA-directed DNA polymerase. However, relatively few

reports of extensive enzyme purification have appeared in which the purity of the DNA polymerase preparation obtained has been rigorously addressed (1 - 4). A contaminant which has proved most difficult to remove from the DNA polymerase preparations is a single-stranded RNA endonucleolytic activity since it appears that trace amounts of such a ribonuclease activity may remain in fractions of even highly purified oncornavirus DNA polymerases (9). This residual amount of RNase activity, which may not be detectable by conventional methods (e.g. RNA solubilization), may be most critical when evaluating the capabilities of the RNA-directed DNA polymerase in vitro in that a single chain scission in the RNA template may completely alter the nature of the true DNA polymerase reaction. In this communication we present evidence indicating that the residual RNase activity routinely contaminating conventionally purified preparations of avian RNA-directed DNA polymerases can be completely eliminated by including low concentrations of bentonite in the reaction mixtures. This inhibition appears selective since little, if any, effect on either the RNA-directed DNA polymerase activity or RNase H activity can be detected in the presence of appropriate concentrations of the inhibitor.

MATERIALS AND METHODS

Reagents. [^{3}H]-thymidine triphosphate (53 Ci/mM) and [^{3}H]-poly (rA) (19 mC/mM) were obtained from Schwarz Bioresearch. [^{3}H]-uridine (20 Ci/mM) was from Nuclear Dynamics. The sources and preparation of other pertinent materials have been described previously (1, 10).

Cells and virus. The B77 strain (subgroup C) of Rous sarcoma virus (RSV) was propagated in duck embryo fibroblasts and purified as previously described (11). The preparation of RSV 70S RNA and [^{3}H]-uridine labeled 70S RNA have been reported previously (11 - 13).

DNA polymerase reaction. Avian myeloblastosis virus (AMV) DNA polymerase, obtained from J.W. Beard of Life Sciences, Inc., St. Petersberg, Florida, through the auspices of the Special Virus Program of the National Cancer Institute was purified as described by Kacian and Spiegelman (14). Two preparations were employed in these studies, V74-17, and V75-2. AMV 70S RNA was purified by previously described methods (11 - 13). Standard DNA polymerase reaction mixtures contained 0.1 M Tris:HCl, pH 8.1; 0.01 M $MgCl_2$; 2% (v/v) 2-mercaptoethanol; 6×10^{-5} M unlabeled deoxynucleoside triphosphates (dATP, dCTP and dGTP); 5.9×10^{-5} M unlabeled TTP; 1×10^{-6}M

[^{3}H]-TTP; 5 μg/ml 70S RNA; 50 units/ml AMV DNA polymerase (1, 15). Reactions (50 μl) were carried out at 37° for 1 hr and the incorporation of radiolabel into acid-precipitable material was determined as previously described (16).

RNase H assay. The RNase H activity was assayed using [^{3}H]-$(rA)_n \cdot (dT)_{12-18}$ as substrate. The components of the standard RNase H reaction mixtures using the AMV DNA polymerase were the same as those for the DNA polymerase reaction described above except that 70S RNA and all dNTPs were omitted and [^{3}H]-$(rA) \cdot (dT)_{12-18}$ included. Reactions (50 μl) were carried out at 37° for 1 hr and the RNase H activity determined as reported previously (17, Collett and Faras, submitted for publication).

Polyacrylamide gel electrophoresis. The procedures used for the analyses of the viral RNA in 2.25% polyacrylamide gels were essentially as those previously published (1). The exact conditions of electrophoresis are described in the legend to Figure 4.

Preparation of bentonite. Bentonite was washed and sorted essentially as previously reported (18).

RESULTS

Effect of bentonite on the DNA polymerase and RNase H activities of RNA-

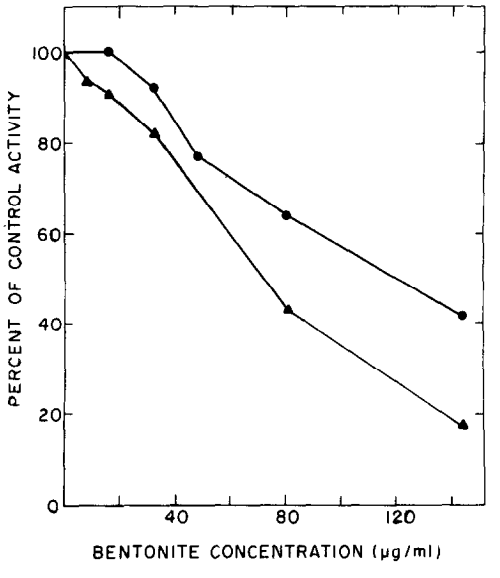


Figure 1. Effect of bentonite on the DNA polymerase and RNase H activities in purified preparations of RNA-directed DNA polymerase. The DNA polymerase and RNase H activities were assayed as described in the Materials and Methods. Each duplicate 50 μl reaction was incubated with the indicated concentrations of bentonite for 1 hr at 37°.

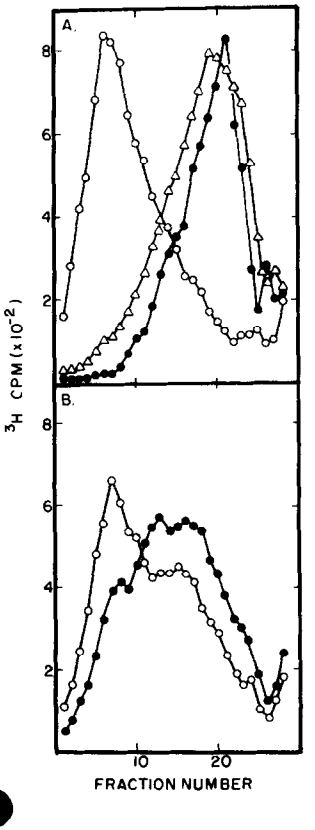
Symbols: ●, DNA polymerase activity
▲, RNase H activity

directed DNA polymerase. Bentonite has been occasionally used during the isolation of RNAs to inhibit general RNase activities (19, 20) and also to selectively remove contaminating RNases in commercial preparations of DNase I and α-amylase (18). Thus, it was of interest to investigate the possible use of bentonite to eliminate residual RNase contamination in preparations of purified RNA-directed DNA polymerase. We therefore investigated first the effect of various concentrations of bentonite on the AMV DNA polymerase activity in reconstructed reactions containing the purified polymerase and purified viral 70S RNA as template. Furthermore, since the oncornavirus-associated RNase H activity is thought to play a role in the synthesis of proviral DNA in vivo (8, 21 - 23) and appears to reside on the same polypeptide as the DNA polymerase activity (3, 24), we also tested the effect of bentonite on this activity. As can be seen in Figure 1, there appears to be a gradual and coordinate inhibition of both the DNA polymerase and RNase H activities with increasing concentrations of bentonite in the reaction mixture.

Effect of bentonite on the integrity of the viral RNA template. To determine if bentonite was capable of eliminating RNase contamination in purified preparations of RNA-directed DNA polymerase, RNase activity was monitored by incubating [^{3}H]-labeled viral 70S RNA in reaction mixtures containing RNA-directed DNA polymerase and varying concentrations of bentonite. After an incubation period of 1 hr, the 70S RNA was denatured and the resulting RNA subunits analyzed by rate-zonal centrifugation (1). The sedimentation profiles of [^{3}H]-labeled viral RNA subunits from such an analysis with one preparation of AMV DNA polymerase is presented in Figure 2. It can be seen that complete RNA degradation occurred in the absence of bentonite with the particular enzyme preparation used. However, upon increasing the concentration of bentonite present in the incubation mixtures, RNA subunit

integrity can be maintained and is nearly completely protected against degrada tion by the enzyme preparation when bentonite is included at 80 µg/ml. Under these conditions, approximately 66% and 45% of the control values of DNA polymerase and RNase H activity, respectively, remained.

As it may be the case that different enzyme preparations purified by identical procedures may vary with respect to purity, and in particular, ribonuclease contamination, we have compared the effects of bentonite on enzymatic activities and RNA template integrity using different preparations of AMV DNA polymerase. The integrity of [^{3}H]-labeled viral RNA subunits after exposure to two different enzyme preparations is compared in Figure 3. The enzyme used to obtain the results depicted in panel B (V75-2) showed no



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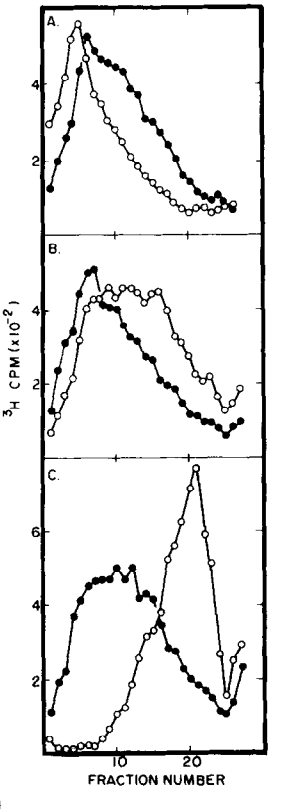

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acid-solubilization of [^{3}H]-labeled 28S ribosomal RNA after a 1 hr incubation period (Table I), but still caused appreciable RNA subunit degradation (Figure 3B). A second enzyme preparation (V74-17) which demonstrated solubilization of radiolabeled RNA (Table I), caused the complete degradation of the high molecular weight viral RNA subunits (Figure 3C). However, when bentonite was included at 32 μg/ml in enzymatic reactions, the integrity of the RNA subunits in both cases was dramatically improved (Figure 3B, C). Under

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Figure 2. Integrity of the viral RNA subunits following incubation in the presence of a purified preparation of AMV DNA polymerase. Incubation mixtures (50 μl), carried out in 0.02 M Tris:HCl, pH 7.4, 0.01M EDTA, contained 5 μg/ml unlabeled 70S RNA, 9000 cpm of [^{3}H]-labeled RSV 70S RNA (from virus harvested at 4 hr intervals), 50 units/ml AMV DNA polymerase (preparation V74-17), and the indicated concentration of bentonite. After 1 hr at 37°C, reaction mixtures were made 0.1M EDTA, 500 μg/ml predigested Pronase, and 0.5% SDS. After 20 min at 37°, nucleic acids were extracted with STE (0.1M NaCl, 0.02M Tris:HCl, pH 7.4, 0.01 M EDTA)-saturated phenol. Samples were then diluted into TE buffer (0.02M Tris:HCl, pH 7.4, 0.01M EDTA), heated to 100° for 60 seconds, and rapidly quenched in ice water. The mixtures were then layered onto 15-30% sucrose gradients and centrifuged in an SW50.1 rotor at 50,000 rpm for 225 min at 4°.

A. Viral RNA subunit profile after incubation in the absence of AMV DNA polymerase, O; incubation in the presence of AMV enzyme, ●; incubation in the presence of AMV enzyme and 8 μg/ml bentonite, Δ.
B. Viral RNA subunit profile after incubation in the presence of AMV enzyme and 32 μg/ml (●) and 80 μg/ml (O) bentonite.

Figure 3. Integrity of the viral RNA subunits following incubation with different preparations of purified AMV DNA polymerase and either the presence or absence of bentonite. Reaction mixtures (50 μl) were as described in the Materials and Methods for the DNA polymerase reaction, except that all dNTPs were omitted and 9000 cpm of [^{3}H]-labeled RSV 70S RNA was included per reaction. Two different preparations of AMV DNA polymerase were analysed as described in the legend to Figure 2 in the presence (32 μg/ml) and absence of bentonite.

A. Viral RNA subunit profile after incubation in the absence of AMV DNA polymerase at 4°, O; at 37°, ●.
B. Viral RNA subunit profile after incubation at 37° in the presence of AMV DNA polymerase (preparation V75-2) with (●) and without (O) bentonite.
C. Viral RNA subunit profile after incubation at 37° in the presence of AMV DNA polymerase (preparation V74-17) with (●) and without (O) bentonite.

TABLE I. RNA solubilization and enzymatic activities in AMV DNA polymerase preparations.

AMV DNA polymerase preparation	percent of control enzymatic activity[a]		percent solubilization of RNA[b]
	DNA polymerase	RNase H	
V74-17	93%	82%	13%
V75-2	78%	63%	0%

[a] DNA polymerase and RNase H activities were determined as described in the Materials and Methods in the presence of 32 μg/ml bentonite and compared to control activities in reactions lacking bentonite.

[b] [^{3}H]-labeled ribosomal 28S RNA was exposed to the enzyme preparations for 1 hr at 37°C. The acid-insoluble material remaining was compared to that in control incubation mixtures lacking enzyme.

these conditions of incubation, both DNA polymerase and RNase H activity are only slightly inhibited (Table I).

In an effort to increase the sensitivity for the detection of limited RNA degradation, similar incubation mixtures as those described in Figure 3 were analyzed by electrophoresis in 2.25% polyacrylamide gels, which affords greater resolution of nucleic acids than rate-zonal sedimentation. As can be seen in Figure 4B, even though there was no solubilization of RNA during the incubation periods employed (Table I), extensive RNA degradation resulted when RNA was incubated in the presence of the purified AMV DNA polymerase (preparation V75-2). However, when bentonite was included at 32 μg/ml with the enzyme, no significant difference in the RNA electrophoretic profile was detectable from that of the control lacking DNA polymerase (Figure 4A, C).

DISCUSSION

The RNA-directed DNA polymerase of oncornaviruses has been purified

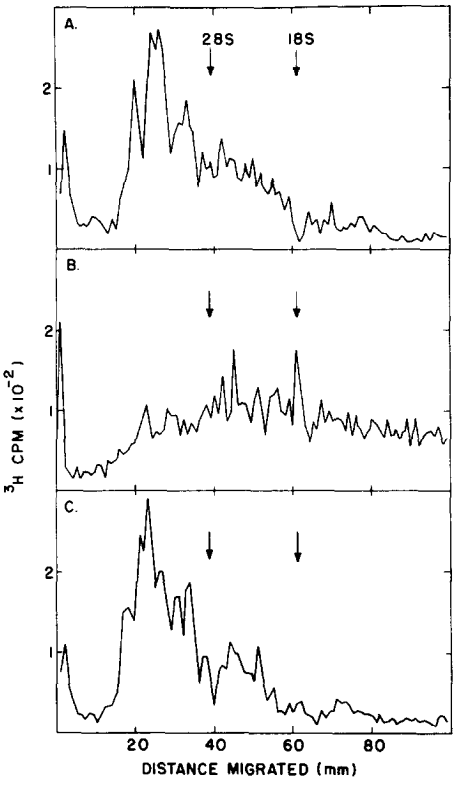

Figure 4. Analysis of the viral RNA subunit integrity by polyacrylamide gel electrophoresis. Reactions (50 µl) were carried out for 1 hr at 37° as described in Figure 3. After Pronase digestion and phenol extraction, mixtures were precipitated with ethanol, centrifuged and the precipitates resuspended in TE buffer (0.2M Tris:HCl, pH 7.4, 0.01M EDTA).

Samples in 100 µl TE buffer were heated to 100° for 60 seconds, quenched, and analyzed by electrophoresis in 6 x 100 mm gels of 2.25% polyacrylamide, 6mA/gel, 4 hr at room temperature. [^{32}P]-labeled 28S and 18S ribosomal RNAs were included as markers. The incubation conditions were as follows:

A. [^{3}H]-labeled viral RNA, 1 hr at 37°.
B. [^{3}H]-labeled viral RNA and AMV DNA polymerase (preparation V75-2), 1 hr at 37°.
C. [^{3}H]-labeled viral RNA and AMV DNA polymerase (preparation V75-2), 1 hr at 37° in the presence of 32 µg/ml bentonite.

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by a variety of techniques. However, when rigorously assayed, trace amounts of ribonuclease activity appear to remain in even highly purified preparations of the DNA polymerase (1, 4, 9). In this communication we report that this contaminating RNA degrading (nicking) activity may be eliminated by including bentonite at low concentrations in incubation mixtures. Under these conditions

the DNA polymerase and RNase H activities of the enzyme preparation are only minimally inhibited. Thus, RNA-directed DNA synthesis may be conducted in the complete absence of template degradation. We are currently investigating the characteristics of the avian RNA-directed DNA polymerase reaction carried out in the presence of bentonite.

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