Mechanism of Action of Moloney Murine Leukemia Virus RNase H III

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The mechanism of action of Moloney murine leukemia virus RNase H III was studied, utilizing the model substrate $(A)_n \cdot (dT)_n$ and polyacrylamide gel electrophoresis to assay enzyme activity. Examination by electrophoresis on 15% polyacrylamide gels in 7 M urea and on DEAE-cellulose paper in 7 M urea revealed that, early in a reaction with $[^3H](A)_n \cdot (dT)_n$ as substrate, RNase H III generated products ranging in length from 80 to 90 nucleotides to <10 nucleotides and that after extended incubation the limit digest products generated were 3 to 15 nucleotides long. Product oligomers were of the following configuration: $[5'-P, 3'-OH](A)_n$. RNase H III was shown to be an exonuclease requiring free ends in its substrate for activity by the inability to degrade RNA inserted in *Escherichia coli* ColE1 plasmid DNA. The enzyme was capable of attacking RNA in RNA-DNA hybrids in the 5' to 3' and 3' to 5' directions as demonstrated by the use of $[^3H, 5'-^{32}P](A)_{600} \cdot (dT)_n$ and cellulose- $[^3H](A)_n \cdot (dT)_n$. RNase H III was random in its mode of action because addition of excess unlabeled $(A)_n \cdot (dT)_n$ to an ongoing reaction with $[^3H](A)_n \cdot (dT)_n$ as substrate resulted in immediate inhibition of enzyme activity.

Study of the RNase H activities present in murine retroviruses has been complicated by the fact that murine virus isolates contain two and sometimes three RNase H species. These have been designated RNase H I (8), RNase H II (5), and RNase H III (4) in their order of identification. RNase H II, characterized in lysates of Moloney murine sarcoma-leukemia virus grown in rat cells, is a random exohydrolase of cellular origin (4; Gerard, unpublished data). RNase H I is the designation given to the murine retrovirus reverse transcriptase-associated RNase H (5, 13, 14, 17; G. F. Gerard, Biochemistry, in press). Good evidence now exists for the virus-coded origin of murine leukemia virus (MLV) RNase H I (11). RNase H III is a smaller enzyme (molecular weight, 30,000) that lacks DNA polymerase activity and appears to be a proteolytic cleavage product of RNase H I (4). Preliminary data consistent with an endonucleolytic mode of action for Moloney MLV (M-MLV) RNase H III have been published (4). This result was unexpected since the putative parent molecule from which RNase H III is derived, RNase H I (4), is clearly an exonuclease (13, 14, 17). I have further characterized the mode of action of M-MLV RNase H III and report that it is a random exonuclease with the unusual ability to generate large as well as small oligonucleotide products.

MATERIALS AND METHODS

The reagents and virus used in this study and the purification of M-MLV RNase H I were as described previously (Gerard, in press).

Purification of M-MLV RNase H III. Because of the extreme instability of M-MLV RNase H III in dilute protein solution (4), a purification procedure was developed that could be completed in 12 h and that yielded enzyme free of most contaminating nuclease activity and having a half-life of approximately 1 month at 4°C. M-MLV (18 mg of protein) was lysed at 6 mg/ml by incubation at 37°C for 30 min in 20 mM Tris-hydrochloride (pH 8.0)-10 mM dithiothreitol-0.2 mM EDTA-10% glycerol-1% sodium deoxycholate-10% Nonidet P-40. The lysate was applied to a 2.5-ml heparin-Sepharose column equilibrated in buffer A (buffer B plus 0.01% Nonidet P-40). After the column was washed with 20 ml of buffer A plus 0.03 M NaCl plus 400 μ g of bovine serum albumin per ml, it was batch eluted with buffer A plus 0.2 M NaCl plus 400 μ g of bovine serum albumin per ml. RNase H I (DNA polymerase) remains bound to the column under these conditions. The peak fractions of RNase H III activity were pooled, and the pool was dialyzed against 1 liter of 20 mM Tris-hydrochloride (pH 8.0)-1 mM dithiothreitol-10% glycerol (buffer B) for 1 h. RNase H III activity in the dialysate was bound to a 1-ml polycytidylic acid-agarose column equilibrated in buffer B. The column was washed with 5 ml of buffer B and 15 ml of buffer B plus 0.02 M KCl, and RNase H III activity was eluted with buffer B plus 0.1 M KCl. Peak fractions of RNase H activity were pooled, and the pool was concentrated by dialysis against 30% polyethylene glycol. The concentrated sample was then dialyzed against buffer B. M-MLV RNase H III purified by this procedure contained no detectable exonuclease activity with the following substrates (5): $[^3H](A)_n$, $[^3H](A)_n \cdot (U)_n$, and $[^3H](dT)_n \cdot (A)_n$.

RNase H assays. Assays for RNase H activity were carried out at 37°C for various times with $[^3H](A)_n \cdot (dT)_n$ and monitored the degradation of

$[^3\text{H}](\text{A})_n$ by various means (4, 5). Standard reaction mixtures (100 μl) contained 20 mM Tris-hydrochloride (pH 8.0)–2 mM dithiothreitol, (dT) $_n$ and radioactivity labeled (A) $_n$ at various concentrations, and KCl and MnCl_2 as indicated below. The (dT) $_n$ used in this study was >1,000 nucleotides long. Assays contained 40 mM KCl and 2 mM MnCl_2 with M-MLV RNase H I, no KCl and 1 mM MnCl_2 with M-MLV RNase H III, 20 mM KCl and 5 mM MgCl_2 with *Escherichia coli* RNase H, and 20 mM KCl and 10 mM MgCl_2 with *E. coli* DNA polymerase I. The reaction conditions used for M-MLV RNase H I and III (4) and *E. coli* RNase H (12) have been established to be optimal for these enzymes with $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ as substrate. One unit of RNase H activity is defined as the amount of enzyme required to solubilize 100 pmol of $[^3\text{H}](\text{A})_n$ in $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ in 20 min at 37°C.

Preparation of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$. $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ [120 μM $[^3\text{H}](\text{A})_n$, 2,000 cpm/pmol; 760 μM (dT) $_n$] was synthesized as described in reference 4.

Preparation of $[^3\text{H}$, 5'- $^{32}\text{P}](\text{A})_{300}$, $[^3\text{H}$, 3'- $^{32}\text{P}](\text{A})_{300}$, $[^3\text{H}](\text{A})_{1,100}$, and cellulose- $[^3\text{H}](\text{A})_n$. $[^3\text{H}$, 5'- $^{32}\text{P}](\text{A})_{300}$ (2,100 cpm/pmol of total phosphate in ^{32}P), $[^3\text{H}$, 3'- $^{32}\text{P}](\text{A})_{300}$ (2,100 cpm/pmol of total phosphate in ^{32}P), $[^3\text{H}](\text{A})_{1,100}$ (2,100 cpm/pmol of total phosphate), and cellulose- $[^3\text{H}](\text{A})_n$ (40 cpm/pmol) were prepared as described previously (Gerard, in press).

Synthesis of $[^3\text{H}$, 5'- $^{32}\text{P}](\text{A})_{600}$. The 5' end of $[^3\text{H}](\text{A})_n$ (Miles Laboratories; 20 cpm/pmol of total phosphate) was dephosphorylated with alkaline phosphatase and rephosphorylated using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (9,000 cpm/pmol) and polynucleotide kinase (9). $[^3\text{H}$, 5'- $^{32}\text{P}](\text{A})_n$ with a specific activity in ^{32}P of 5 cpm/pmol of total phosphate and an average chain length of 600 was obtained. The chain length of the product was calculated (2) from a sedimentation coefficient (8.0S) determined in 1 M NaCl, with (A) $_{100}$ (4.3S) as a marker.

(A) $_n$. Oligomers of (A) $_n$ used as standards for electrophoresis on 7 M urea-polyacrylamide gels and DEAE-cellulose paper were obtained from various sources. A(A) $_2$, A(A) $_3$, A(A) $_4$, A(A) $_5$, A(A) $_6$, A(A) $_7$, A(A) $_8$, and A(A) $_9$ were purchased from Collaborative Research Inc. A $_{16}$ (1.5S), A $_{24}$ (2.6S), and A $_{33}$ (3.3S) were obtained from Miles Laboratories, Inc. $[^3\text{H}](\text{A})_{33}$ (2.1S), $[^3\text{H}](\text{A})_{24}$ (1.8S), $[^3\text{H}](\text{A})_{17}$ (1.5S), and $[^3\text{H}](\text{A})_{10}$ (1.2S) were prepared as described previously (Gerard, in press).

7 M urea-polyacrylamide disc gel electrophoresis. Electrophoresis on 15% polyacrylamide gels containing 7 M urea for examination of the size distribution of labeled polymers after digestion with RNase H was carried out as described previously (4, 9). The position of migration of unlabeled (A) $_n$ was determined by staining gels with Stains-All (Bio-Rad Laboratories) (1). Gels containing radioactively labeled (A) $_n$ were fractionated by cutting into 2-mm slices (4, 9). Radioactivity was eluted from gel slices by incubation at 37°C in a toluene-base scintillation fluid containing 3% (vol/vol) Protosol (New England Nuclear Corp.). A total of 60 to 80% of the radioactivity applied to gels was recovered during this procedure.

Generation of acid-soluble products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ for analysis by paper electrophoresis

and chromatography. One-milliliter standard reaction mixtures containing 1.2 nmol of $[^3\text{H}](\text{A})_n$ (2,000 cpm/pmol) and 7.6 nmol of (dT) $_n$ were incubated for 40 min with either 12.5 U of RNase H I or 13.9 U of RNase H III. RNase H I solubilized 93% and RNase H III solubilized 75% of the $[^3\text{H}](\text{A})_n$ present. Acid-insoluble $[^3\text{H}](\text{A})_n$ was removed by precipitation with 15% trichloroacetic acid, and the acid was neutralized with NaOH.

Portions (100 μl) of the $[^3\text{H}](\text{A})_n$ digestion products were degraded with alkali (20 μl of 2 N KOH) by incubating at 37°C for 18 h. KOH was neutralized by the addition of 10 μl of 4 N perchloric acid, and potassium perchlorate was removed by centrifugation.

Portions (50 μl) of the $[^3\text{H}](\text{A})_n$ digestion products were degraded enzymatically during a 1-h incubation by the addition of 50 μl of 0.03 M MgCl_2 –0.2 M glycine-NaOH (pH 9.1) and 1 U of snake venom phosphodiesterase.

Paper chromatography. The snake venom phosphodiesterase-digested, acid-soluble digestion products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ were spotted on a 56-cm strip of Schleicher Schuell no. 589 orange ribbon-C paper along with 200 μg each of adenosine and 5'-AMP. Chromatography was in *n*-propanol- NH_4OH -water (55:10:35, vol/vol/vol) for 20 to 24 h. The position of standards was located by UV light. Strips (1 cm) were cut, and radioactivity was determined by scintillation spectroscopy.

Paper electrophoresis. The alkali-digested, acid-soluble digestion products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ were spotted on a 56-cm strip of Schleicher Schuell no. 589 orange ribbon-C paper along with 200 μg each of adenosine, 3'-AMP, and 3',5'-ADP. Electrophoresis was in 0.03 M KH_2PO_4 (pH 7.3) for 2 h at 1 kV. The position of standards was determined with UV light, and strips (1 cm) were cut for determination of radioactivity by scintillation spectroscopy.

The distribution of the oligomers of adenosine in the acid-soluble digestion products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ was determined by electrophoresis on Whatman DEAE-cellulose paper in 0.05 M potassium phosphate (pH 7.5) and 7 M urea. 5'-AMP (50 μg) and A(A) $_2$, A(A) $_3$, A(A) $_4$, A(A) $_5$, A(A) $_6$, A(A) $_7$, and A(A) $_9$ (absorbance at 260 nm, 0.2 to 0.7 units each) were spotted with each sample. Electrophoresis was for 4.5 h at 1 kV. The position of standards was determined with UV light. Strips (0.5 or 1 cm) were cut, and radioactivity was determined by scintillation spectroscopy.

RESULTS

Comparison of the digestion products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ generated by M-MLV RNase H I and III. Utilizing electrophoresis in accurately standardized 7 M urea–25% polyacrylamide gels, M-MLV RNase H I was shown to degrade $[^3\text{H}](\text{A})_{1,100} \cdot (\text{dT})_n$ to oligomers of (A) $_n$ with a cross section of lengths ranging from 26 to <10 nucleotides (Gerard, in press). Previous estimates of the size of oligomers generated by the action of RNase H III on $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ after approximately 10% of the available $[^3\text{H}](\text{A})_n$ was solubilized indicated that RNase H III produces oligomers several times longer than

this (4). The gels used in these studies were not adequately standardized with reference markers, so product sizes could not be determined accurately. Therefore, 7 M urea-15% polyacrylamide gels were standardized with various species of $(A)_n$ where $10 \leq n \leq 90$. The products generated by the action of RNase H III on $[^3H, 5'-^{32}P](A)_{380} \cdot (dT)_n$ during a 20-min reaction were analyzed by electrophoresis in 7 M urea-15% polyacrylamide gels (Fig. 1). A ratio of substrate to enzyme was selected so that, even after 20 min of incubation, 57% of the 3H counts and 45% of the ^{32}P counts were still present in $[^3H, 5'-^{32}P](A)_n$ of >100 nucleotides in length. Early in the reaction (Fig. 1B), products ranging in length from approximately 80 to 90 nucleotides down to <10 nucleotides were generated. The enzyme might have generated products longer than 90 nucleotides, but such products could not be differentiated from undigested substrate in this gel system. As the reaction proceeded, products of all sizes continued to be produced, although between 10 and 20 min the amounts of larger oligonucleotides of $(A)_n$ ($n > 35$) appeared to decrease, particularly those labeled with ^{32}P . A more quantitative appraisal of the kinetics of degradation in Fig. 1 was performed by calculating the relative average total number of molecules and 5'-end-derived molecules in various product size classes [e.g., $(A)_{84}$, $(A)_{51}$, $(A)_{28}$, A_{18} , and A_8] produced with time (data not shown). The results indicated that at 2.5 min products of all size classes were produced and that the relative total number of products exceeded the number of 5'-end-derived products by approximately fivefold in each size class. After 20 min of incubation, this increased to a 10-fold difference in the large size classes [i.e., $(A)_{84}$, $(A)_{51}$, and $(A)_{28}$] and a 20-fold difference in the small size classes [i.e., $(A)_{18}$ and $(A)_8$]. In the small size classes, the total number of $(A)_n$ products as well as the number of 5'-end-derived products increased at linear rates throughout the course of the reaction. Within 5 min after the start of the reaction, the rates of formation of 5'-end-derived $(A)_n$ products in the larger size classes leveled off and actually began to decrease slightly. The rates of formation of the total population of $(A)_n$ products in the larger size classes leveled off after 10 min and increased slightly thereafter. These results suggest that, as a reaction proceeds, larger size products continue to serve as substrate, being repeatedly cleaved to progressively smaller material. As a control, $[^3H, 5'-^{32}P](A)_{380}$ alone was incubated with RNase H III under standard assay conditions. No 3H or ^{32}P was released from $[^3H, 5'-^{32}P](A)_{380}$ into smaller material in the absence of $(dT)_n$.

In spite of the dramatic difference in length

FIG. 1. Polyacrylamide gel electrophoresis in 7 M urea of digestion products of $[^3H, 5'-^{32}P](A)_{380} \cdot (dT)_n$ produced by M-MLV RNase H III. Standard RNase H reaction mixtures contained 240 pmol of $[^3H, 5'-^{32}P](A)_{380}$, 290 pmol of $(dT)_n$, and 0.09 U of RNase H III. Reaction mixtures were incubated for 0 (A), 2.5 (B), 10 (C), and 20 (D) min. Polyacrylamide gels (15%) were analyzed for 3H (O) and ^{32}P (●) radioactivity (see text). The arrows show the position of migration of standards of $(A)_n$ of various chain lengths.

between the initial cleavage products of $(A)_n$ generated by RNase H I (Gerard, in press) and RNase H III (Fig. 2), Fig. 3 illustrates that, when

the reaction was permitted to proceed for a long period of time (40 min) with an excess of either enzyme (approximately 13 U), most of the product became acid soluble, and the size distribution of the products generated was quite similar when analyzed by DEAE-cellulose paper electrophoresis in 7 M urea. Forty to 50% of the products were 2 to 8 nucleotides long, whereas the remainder was ≥ 9 nucleotides long, but probably <15 nucleotides long.

M-MLV RNase H I produces digestion products with 5'-phosphate and 3'-OH ends (17). To test whether M-MLV RNase H III generated products with the same termini, two experiments were performed (17). Acid-soluble digestion products of $[^3\text{H}](\text{A})_n$ produced by RNase H I and III were subjected to digestion by snake venom phosphodiesterase. Paper chromatography of the digestion products revealed that phosphodiesterase converted 87 and 82%, respectively, of the RNase H I and III digestion products to 5'-AMP (Table 1). Snake venom phosphodiesterase is a 3'-exonuclease which requires a 3'-OH end for activity and generates mononucleotides with a 5'-phosphate (3, 15). Approximately 10% of the radioactivity migrated with adenosine in each case, indicating that the snake

FIG. 2. Comparison of the digestion products of $[^3\text{H}](\text{A})_{100} \cdot (\text{dT})_n$, produced by M-MLV RNase H III (A) and RNase H I (B) by electrophoresis in 7 M urea-15% polyacrylamide gels. Standard RNase H reaction mixtures incubated for 5 min contained 1,250 pmol of $[^3\text{H}](\text{A})_{100}$ (20 cpm/pmol), 1,875 pmol of $(\text{dT})_n$, and no enzyme ($\bullet$), 0.9 U of RNase H III ($\circ$), or 0.6 U of RNase H I ($\circ$). The arrows show the position of migration of standards of $(\text{A})_n$ of various chain lengths.

FIG. 3. DEAE-cellulose paper electrophoresis in 7 M urea of the acid-soluble digestion products of $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ generated by M-MLV RNase H I (A) and RNase H III (B). Products were generated and electrophoresis was performed as described in the text.

venom phosphodiesterase was contaminated with a small amount of phosphatase activity. To exclude the possibility that the contaminating phosphatase activity might have contributed to digestion of the oligonucleotides by first dephosphorylating their 3' ends, the RNase H I and III digestion products were subjected to alkaline hydrolysis. Electrophoresis of alkaline digests revealed the presence of radioactivity in approximately equal amounts which comigrated with adenosine and authentic pAp (Table 2). Adenosine and pAp in equal amounts could have been generated by alkaline hydrolysis only if the digestion products had 5'-phosphate and 3'-OH termini. Therefore, the results from both experiments demonstrate that M-MLV RNase H III resembles RNase H I in generating digestion products with 5'-phosphate and 3'-OH ends.

Mode of action of M-MLV RNase H III: endonucleolytic or exonucleolytic. M-MLV RNase H I is an exonuclease that requires free ends in an RNA-DNA substrate for activity, based upon its inability to cleave RNA inserted in one strand of the double-stranded superhelical *E. coli* plasmid DNA, ColE1 (13, 17). The assay utilizing radioactively labeled ColE1 DNA involves monitoring the conversion of 23S superhelical DNA to a 17S open circular form by

TABLE 1. Paper chromatography of snake venom phosphodiesterase digests of acid-soluble products generated from [^3H](A) $_n$ ·(dT) $_n$ by RNase H I and III^a

Enzyme	³ H cpm		
	Origin	5'-AMP	Adenosine
RNase H I	172 (2.7) ^b	5,578 (86.8)	677 (10.5)
RNase H III	428 (7.9)	4,421 (82.1)	536 (10.0)

^a Generation of acid-soluble products of [^3H](A) $_n$ ·(dT) $_n$, snake venom phosphodiesterase digestion, and paper chromatography were performed as described in the text.

^b Numbers in parentheses indicate the percentage of total radioactivity present.

TABLE 2. Paper electrophoresis of alkaline digests of acid-soluble products generated from [^3H](A) $_n$ ·(dT) $_n$ by M-MLV RNase H I and III^a

Enzyme	³ H cpm		
	Adenosine	3'-AMP	3',5'-ADP
RNase H I	995 (19.3) ^b	3,177 (61.4)	1,000 (19.3)
RNase H III	647 (17.3)	2,445 (65.4)	649 (17.3)

^a Generation of acid-soluble products of [^3H](A) $_n$ ·(dT) $_n$, alkaline digestion, and paper electrophoresis were performed as described in the text.

^b Numbers in parentheses indicate the percentage of total radioactivity present.

sucrose gradient centrifugation (4) or by agarose slab gel electrophoresis (10).

Based upon analyses of digestion products of [^3H](A) $_n$ ·(dT) $_n$, such as those described in Fig. 1 and 2, it was tentatively concluded that Moloney murine sarcoma-leukemia virus RNase H III functions endonucleolytically (4). The DNase contamination in the preparations of RNase H III used in the previous studies was such that no exonuclease activity could be detected with [^3H](dT) $_n$ ·(A) $_n$ (2,200 cpm/pmol), but analysis of mode of action with ColE1 DNA was precluded by the presence of contaminating DNA endonuclease (4). However, the DNA endonuclease contamination levels in M-MLV RNase H III purified by the procedure used here (see above) were reduced to the extent that analysis of the mode of action could be carried out with the *E. coli* ColE1 [^3H]DNA containing inserted RNA. Under standard assay conditions, 1.1 U of M-MLV RNase H III converted 9% of supercoiled ColE1 [^3H]DNA (0.5 μg) to the open circular form in 1 h. Exposure of 0.3 μg of simian virus 40 supercoiled DNA, which contains no inserted RNA, to 2 U of M-MLV RNase H III under the same assay conditions resulted in the conversion of a comparable amount of supercoiled DNA to the open circular form, suggesting

that the small amount of conversion observed with ColE1 DNA was due to a small amount of DNA endonuclease contamination in M-MLV RNase H III. *E. coli* RNase H (1.4 U), which is an endonuclease (12), converted 42% of the ColE1 [^3H]DNA under standard assay conditions in 1 h. Based upon this type of analysis, M-MLV RNase H III requires free ends in its substrate for activity and, therefore, can be classified as an exonuclease. The experiments described below with double-labeled substrates confirm this conclusion.

An end-labeled substrate such as [^3H , 5'- ^{32}P](A) $_{600}$ ·(dT) $_n$ can be used to demonstrate that a particular species of RNase H is not an endonuclease, when the kinetics of acid-soluble product formation are determined (Fig. 4). An endonuclease such as *E. coli* RNase H (12) solubilized ^{32}P and ^3H at the same rate with [^3H , 5'- ^{32}P](A) $_{600}$ ·(dT) $_n$ as substrate (Fig. 4B), whereas a random 5'- to 3'-exonuclease such as *E. coli* DNA polymerase I solubilized ^{32}P at a much faster rate than ^3H (Fig. 4C). M-MLV RNase H III solubilized ^{32}P at a faster rate than ^3H (Fig. 4A), consistent with a 5'- to 3'-exonucleolytic mode of attack.

M-MLV RNase H III can also attack the RNA in an RNA-DNA hybrid from the 3' terminus,

FIG. 4. Degradation of [^3H , 5'- ^{32}P](A) $_{600}$ ·(dT) $_n$ by M-MLV RNase H III. Standard RNase H reaction mixtures containing 450 pmol of [^3H , 5'- ^{32}P](A) $_{600}$ and 750 pmol of (dT) $_n$ were incubated for the times indicated. Reaction mixtures also contained: (A) RNase H III, 0.5 U; (B) *E. coli* RNase H, 0.17 U; (C) *E. coli* DNA polymerase I, 500 μg of protein. Time courses of the percent ^3H (○) and ^{32}P (●) solubilized were determined.

as demonstrated by its ability to degrade $[^3\text{H}](\text{A})_n$ in cellulose- $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ (Table 3), where the $[^3\text{H}](\text{A})_n$ is blocked at the 5' end. M-MLV RNase H III therefore resembles M-MLV RNase H I (Gerard, in press) in being an exonuclease with the ability to attack a substrate in either the 5' to 3' or 3' to 5' direction.

Mode of action of M-MLV RNase H III: random or processive exonucleolytic. A processive exonuclease, once bound to a polynucleotide chain substrate, does not dissociate until the substrate chain is degraded completely. A random exonuclease dissociates from the substrate molecule after each hydrolytic event. M-MLV RNase H I is an example of an exohybridase that is partially processive (Gerard, in press) and does not dissociate after each hydrolytic event but rather stays bound to a substrate through a finite, average number of catalytic events. One method used to differentiate a processive from a random exonuclease is substrate competition. The kinetics of degradation of $[^3\text{H}](\text{A})_{1,100} \cdot (\text{dT})_n$ catalyzed by M-MLV RNase H III were determined in the absence and presence of a 10-fold excess of unlabeled $(\text{A})_n \cdot (\text{dT})_n$ added 1 min after the start of a reaction. The amount of product of all sizes formed was determined by electrophoresis in 7 M urea-15% polyacrylamide gels. Figure 5 illustrates that the degradation of $[^3\text{H}](\text{A})_{1,100} \cdot (\text{dT})_n$ by M-MLV RNase H III was almost totally inhibited after excess unlabeled substrate was added to an ongoing reaction. These results are most consistent

FIG. 5. Effect of addition of excess $(\text{A})_n \cdot (\text{dT})_n$ on the rate of degradation of $[^3\text{H}](\text{A})_{1,100} \cdot (\text{dT})_n$ by M-MLV RNase H III. $[^3\text{H}](\text{A})_{1,100} \cdot (\text{dT})_n$ (960 pmol), $(\text{dT})_n$ (1,160 pmol), and 0.46 U of M-MLV RNase H III were added to three sets of standard scaled-up (fourfold) reaction mixtures. Unlabeled $(\text{A})_n$ (10.4 nmol) and $(\text{dT})_n$ (12.4 nmol) were either not added (○), added before the start of the reaction (□), or added 1 min after the start of the reaction (●). Samples (100 μl) were withdrawn at various times and analyzed by electrophoresis on 15% polyacrylamide gels. The total radioactivity from each gel present in $[^3\text{H}](\text{A})_n$ smaller than 70 nucleotides is plotted.

with RNase H III functioning as a random exonuclease.

DISCUSSION

Based upon the observation that RNase H III produced large oligonucleotide products, the enzyme was classified previously as an endonuclease (4). Results from several independent experimental approaches presented in this report demonstrate that RNase H III is not endonucleolytic, but rather is an exonuclease requiring free ends in its substrate for activity. The enzyme did not readily cleave RNA contained in the circular superhelical DNA, ColE1. RNase H III solubilized the ^{32}P in $[^3\text{H}, 5'\text{-}^{32}\text{P}](\text{A})_{380} \cdot (\text{dT})_n$ at a faster rate than ^3H , indicative of a preferred exonucleolytic attack at the 5' end of the substrate. $[^3\text{H}](\text{A})_n$ blocked at the 5' end with cellulose was also degraded by RNase H III in the presence of $(\text{dT})_n$, suggesting that in addition to attacking in the 5' to 3' direction, the enzyme can also attack at the 3' end of a substrate. Exonucleolytic attack on $[^3\text{H}, 5'\text{-}^{32}\text{P}](\text{A})_{380} \cdot (\text{dT})_n$ by RNase H III in both directions resulted in a faster rate of solubilization of ^{32}P than ^3H since only the ^{32}P was present strictly at the end of the substrate chain.

RNase H III was quickly trapped by the addition of an excess of unlabeled $(\text{A})_n \cdot (\text{dT})_n$ to an ongoing reaction, demonstrating that the enzyme readily dissociates from the substrate, probably after each hydrolytic event. RNase H III therefore appears to preferentially attack the ends of substrate molecules in a random fashion,

TABLE 3. Test of the ability of RNase H III to degrade $[^3\text{H}](\text{A})_n$ blocked at the 5' terminus^a

Enzyme	% $[^3\text{H}](\text{A})_n$ solubilized from cellulose- $[^3\text{H}](\text{A})_n$	
	-(dT) _n	+(dT) _n
M-MLV RNase H III	5	21
M-MLV RNase H I	6	31
<i>E. coli</i> RNase H	1	23

^a $[^3\text{H}](\text{A})_n$ was covalently attached at its 5' end to cellulose as described in the text. A suspension (0.1 ml) of cellulose- $[^3\text{H}](\text{A})_n$ (9,200 cpm of ^3H ; 40 cpm/pmol) was centrifuged for 4 min in a Microfuge B (Beckman Instruments, Inc.), and the supernatant was discarded. The cellulose was suspended in standard reaction mixtures (100 μl) containing 1,050 pmol of $(\text{dT})_n$, where indicated, and one of the following: 1.3 U of RNase H I, 0.2 U of RNase H III, or 10 U of *E. coli* RNase H. During a 20-min incubation, tubes were mixed every 5 min. The reaction was stopped by the addition of 200 μl of cold water and centrifuged as above. The supernatant was counted in aqueous scintillation fluid. The amount of $[^3\text{H}](\text{A})_n$ released by micrococcal nuclease (50%) was assumed to be all the $[^3\text{H}](\text{A})_n$ accessible to attack by the enzymes.

similar to avian myeloblastosis virus α RNase H (9; Gerard, in press).

Early in a reaction with $[^3\text{H}](\text{A})_n \cdot (\text{dT})_n$ as substrate, all of the digestion products of adenosine generated by M-MLV RNase H I were less than 26 nucleotides long (Fig. 2; Gerard, in press). In contrast, many of the early digestion products made by RNase H III were much longer, i.e., 30 to 90 nucleotides long (Fig. 1 and 2). After extensive degradation, both enzymes generated products with a similar length distribution (range, 2 or 3 to approximately 14 nucleotides). M-MLV RNase H III resembled RNase H I in generating products with 3'-OH and 5'-phosphate termini.

Based on size analysis studies of the ^{32}P -labeled degradation products derived from the 5' end of $[^3\text{H}, 5'\text{-}^{32}\text{P}](\text{A})_{380}$ generated early in a reaction by RNase H III (Fig. 1B), the number of nucleotides from the end of the $(\text{A})_n$ chain at which the enzyme makes a cut could be any number between 3 and 90. An exonucleolytic mode of attack accompanied by cleavage of the RNA-DNA substrate as far as 90 nucleotides away from the $(\text{A})_n$ terminus is difficult to reconcile. Formation of these longer polynucleotide products could be explained in part if RNase H III were capable of binding to and attacking $(\text{A})_n \cdot (\text{dT})_n$ hybrid termini composed of an end of $(\text{dT})_n$ overlapped by various chain lengths of $(\text{A})_n$. However, RNase H III also generates large products with a substrate such as fd DNA- ^3H RNA (16), in which there is no slippage of the RNA and DNA strands (Gerard, unpublished data), suggesting that this mechanism cannot account for all of the long $(\text{A})_n$ products formed. A possible trivial explanation is that the large fragments observed are not cleavage products, but are partially degraded chains of starting substrate from which smaller oligomers have been cleaved. This interpretation might explain some of the large fragments present late in a reaction, but not those formed early. The relative total number of small fragments [i.e., $(\text{A})_{28}$, $(\text{A})_{18}$, and $(\text{A})_8$] present at 2.5 min of incubation is too small by an order of magnitude to account for the relative total number of large fragments [i.e., $(\text{A})_{34}$ and $(\text{A})_{34}$] observed, assuming that the large fragments represent undigested substrate derived from $(\text{A})_{380}$ by the loss of smaller digestion products. Other possibilities can be postulated, none of which has experimental support at this time. For example, the enzymatically active form of RNase H III could have two active sites that function in concert; one site could bind at the end of the substrate molecule, facilitating cleavage of the substrate at some point distal to its terminus by the second active site.

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