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Ribonuclease H activities associated with viral reverse transcriptases are endonucleases

(human immunodeficiency virus/avian myeloblastosis virus)

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ABSTRACT **A series of test substrates have been synthesized to establish the effect of termini on the putative exoribonuclease H activity of reverse transcriptase. Recombinant reverse transcriptase from human immunodeficiency virus, natural enzyme from avian myeloblastosis virus, and a known endonuclease, *Escherichia coli* ribonuclease H, cleaved relaxed, circular, covalently closed plasmids in which 770 consecutive residues of one strand were ribonucleotides. The avian enzyme also deadenylated capped globin mRNA with a covalently attached oligo(dT) tail at the 3′ end. These results resolve a long-standing controversy—that the viral enzymes are obligatory exonucleases *in vitro*, based on their failure to cleave certain substrates for *E. coli* ribonuclease H, including circular poly(A)·linear poly(T) and ribonucleotide-substituted supercoiled plasmids, but resemble endonucleases *in vivo*, based on their ability to degrade RNA in complex DNA·RNA hybrids. The data strongly suggest that the viral enzymes are endonucleases with exquisite sensitivity to the conformation of heteroduplexes. Inhibition of viral, but not cellular, ribonuclease H with ribonucleoside-vanadyl complexes further distinguishes these enzymes.**

The functions encoded in the genomes of retroviruses have come under increasingly intense scrutiny since the human immunodeficiency virus (HIV) was isolated and recognized as the causative agent of AIDS. However, the most promising drugs or vaccines target only two HIV functions; 3′-azido-3′-deoxythymidine (AZT) acts predominantly as an inhibitor of the polymerase activity of reverse transcriptase (1) and recombinant CD4 inhibits viral attachment by binding to products of the *env* gene (reviewed in ref. 2).

A retroviral function that has attracted relatively little attention, despite its being carried out by one of the better characterized proteins, is the ribonuclease H (RNase H) activity of reverse transcriptase. An RNase H cleaves RNA in a DNA·RNA hybrid. The viral enzyme is responsible for degradation of genomic viral RNA after synthesis of minus-strand DNA has occurred. Upon removal of the RNA moiety of the DNA·RNA hybrid, minus-strand DNA becomes available as a template for synthesis of plus-strand DNA (3). The RNase H activity of reverse transcriptase has also been implicated in releasing the tRNA primer that is covalently bound to minus-strand DNA (4), in nicking viral RNA to generate the polypurine-rich oligoribonucleotide that primes synthesis of plus-strand DNA, and in removing the polypurine tract from the DNA extension to which it is linked (5–9).

The RNase H activities of reverse transcriptases are believed to be exonucleases that can attack RNA from either the 5′ or the 3′ end, whereas the normal cellular RNase H activities are endonucleases (10–12). These conclusions were based on experiments with molecules of two types: synthetic hybrids with blocked termini and circles. Both were cleaved by the RNase H isolated either from *Escherichia coli* or from mammalian tissues, but neither served as a substrate for the viral enzymes. Clearly, an AIDS drug might be designed to exploit this difference.

We report here a comparative study of the effect of termini on a recombinant HIV reverse transcriptase, the natural enzyme from avian myeloblastosis virus (AMV), and *E. coli* RNase H. Because these enzymes cleaved our constructs, including covalently closed circular DNA with 770 consecutive ribonucleotide substitutions, the conclusion must be drawn that the RNase H activities of reverse transcriptases are endonucleases.

EXPERIMENTAL PROCEDURES

Materials. The sources of enzymes, nucleic acids, and accessories were as follows: AMV reverse transcriptase, Molecular Genetic Resources (Tampa, FL); recombinant HIV reverse transcriptase, Genetics Institute (Cambridge, MA); T4 RNA ligase, T4 polynucleotide kinase, *E. coli* RNase H, *E. coli* poly(A) polymerase, and rabbit globin mRNA containing 30% rRNA on a molar basis (13), BRL; endonuclease-free *E. coli* DNA polymerase I (Pol I), Boehringer Mannheim; *Micrococcus luteus* polynucleotide phosphorylase (type 15), T4 DNA ligase and oligomers, Pharmacia; DNase I, T7 RNA polymerase, pGEM-4, and RNasin, Promega; pancreatic RNase A, Sigma; restriction enzymes and mung bean nuclease, New England Biolabs; and Bluescript, Stratagene. Radiolabeled compounds came from Amersham.

Preparation of 3′-End-Modified Globin mRNA. Globin mRNA with one labeled adenylate residue at the 3′ end was synthesized as described (13). Subsequently, reactions catalyzed by RNA ligase were performed with 27 pmol (1.4×10^6 dpm) of mRNA and 125 pmol of cytidine 5′,3′-bisphosphate (pCp) in 25 μl, or with 12.5 pmol (10^7 dpm) of mRNA and 370 pmol of $p(dT)_{17}$ in 10 μl, under conditions specified by England *et al.* (14) for large viral RNAs but without dimethyl sulfoxide. Unreacted globin mRNA was removed from mRNA-$p(dT)_{17}$ by diluting 1:2 and treating with polynucleotide phosphorylase (15). Both modified mRNAs were purified by phenol/chloroform extractions (16) and drop dialysis against H_2O (17); this procedure completely eliminated excess pCp and $p(dT)_{17}$. Similar reactions were also carried out with unlabeled reagents, and the stated specific activities were achieved by mixing labeled and unlabeled materials. Yields of mRNA-pCp and mRNA-$p(dT)_{17}$ were ≈95% and 100%, respectively. They were ascertained by performing ligations of measured amounts of unlabeled globin mRNA with either $[^{32}P]$pCp or $[^{32}P]p(dT)_{17}$ of known specific activ-

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Abbreviations: AMV, avian myeloblastosis virus; HIV, human immunodeficiency virus; Pol I, polymerase I.

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ity, under the conditions above, and determining the amount of radioactivity rendered Cl_3CCOOH precipitable.

Preparation of Unligated and Ligated Hybrids. Both ligated and unligated hybrids consist of three molecules: a 770-base labeled RNA, covalently closed circular single-stranded Bluescript DNA containing 770 bases complementary to the RNA ("innertube" DNA), and single-stranded linear Bluescript with sequences complementary to the entire circular Bluescript DNA, excepting the RNA-derived region ("outertube" DNA). The construction of both hybrids is illustrated in Fig. 1.

The plasmid, pGEM-4.723, was generated from pGEM-4 by the insertion of a 723-base-pair *Pst* I fragment of a porcine major histocompatibility complex gene into the *Pst* I site (18). Using Promega's instructions, RNA was transcribed with T7 RNA polymerase in a 20-μl reaction mixture, containing 150 μM UTP (including 50 μCi of RNA-grade [α-^{32}P]UTP; 1 Ci = 37 GBq) and 40 ng of the gel-purified *Pvu* II–*Sma* I fragment of pGEM-4.723. DNA was digested with DNase I and the 5′-triphosphate was removed (19). The RNA was phenol/chloroform extracted and drop-dialyzed as described above; afterwards, it was phosphorylated (19), ethanol-precipitated, and dissolved in 10 μl of water. The product was predominantly 0.77 kilobase in denaturing gels (data not shown) but a component at 1.5 kilobases was also observed. The latter molecule is ribonuclease-resistant and is probably a double-stranded RNA hairpin.

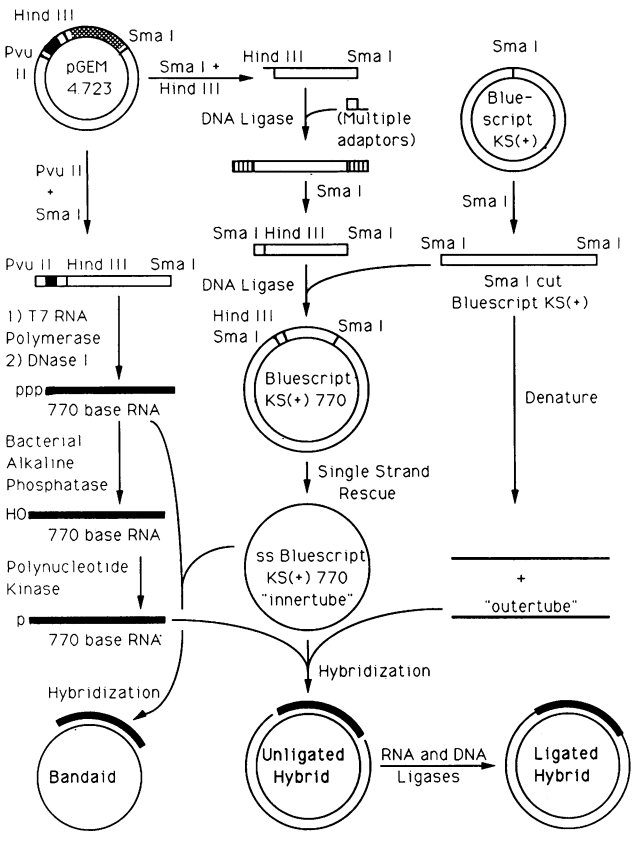

FIG. 1. Scheme for the construction of circular molecules. Three circular molecules were synthesized: (*i*) bandaid, consisting of radioactive RNA hybridized to single-stranded DNA containing sequences complementary to the entire RNA; (*ii*) unligated hybrid, a perfectly base-paired molecule consisting of the single-stranded circular DNA in bandaid hybridized both to monophosphoryl RNA and to linear DNA complementary to all circular DNA sequences with the exception of those hybridized to RNA; and (*iii*) ligated hybrid, the unligated hybrid with the nicks sealed to make a covalently closed circular molecule. The 723-base-pair major histocompatibility complex insert in the *Pst* I site of pGEM-4 (*Top left*) is indicated by ▩. Although not explicitly shown, major histocompatibility complex sequences are also found in all molecules in the *left* and *center* arrays as well as in the ligated hybrid (*Bottom right*). The adaptor (*Top center*) includes DNA from the T7 promoter, represented by ■, to the *Hin*dIII site; multiple adaptors ligated to the same fragment are illustrated by closely spaced vertical lines without labels. Radioactive RNA and unlabeled DNA are indicated by thick and thin solid lines, respectively. A fourth circular molecule, modified bandaid (cartoon not shown), consists of the RNA moiety found in ligated and unligated hybrids and the DNA component of bandaid.

Covalently closed circular single-stranded DNA containing an insert complementary to the RNA transcript was constructed from double-stranded Bluescript KS(+) and two pieces of DNA: a double-stranded adaptor, 5′-GGGAGAC-CGGA-3′, hybridized to 3′-CCCTCTGGCCTTCGA-5′, representing the region from the transcriptional start site to the *Hin*dIII site; and the *Hin*dIII–*Sma* I fragment of pGEM-4.723. Adaptors, in 100-fold excess, were ligated to the *Hin*dIII–*Sma* I fragment and the excess was removed with *Sma* I. This construct was ligated into *Sma* I-cleaved Bluescript. After selecting the correct orientation, the desired strand of Bluescript KS(+)770 was rescued using the Stratagene procedure.

Linear Bluescript was obtained by cleaving double-stranded Bluescript KS(+) with *Sma* I; strands were separated by denaturation as described below.

To assemble unligated hybrids, 5 μg of circular single-stranded innertube DNA and 12.5 μg of linear Bluescript KS(+) DNA, one strand of which is outertube, were boiled for 2 min in 130 μl of 65% (vol/vol) deionized formamide/40 mM Pipes·NaOH, pH 6.5/0.4 M NaCl. After cooling to 70°C, 1.5 μg (5×10^7 dpm) of RNA was allowed to anneal for 1 hr as the mixture was cooled to 55°C. Hybrids were ethanol-precipitated, dissolved in 50 μl of H_2O, and drop-dialyzed.

Ligated hybrids were obtained by incubating 45 μl of unligated hybrids overnight at 15°C with 160 units of RNasin, 35 units of T4 DNA ligase, and 16 μg of T4 RNA ligase in 150 μl of buffer composed of 50 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 250 mM ATP. After several phenol/chloroform extractions, hybrids were drop-dialyzed against H_2O. Where indicated, nicked circular molecules were removed by treatment with endonuclease-free Pol I for 75 min under the conditions detailed in the legend to Fig. 3. The product was repurified by using organic solvents and dialysis as described above.

Synthesis of "Bandaid." Bandaid, consisting of labeled RNA hybridized to single-stranded circular Bluescript KS(+)770, was prepared using the methods above for unligated hybrids except that the linear DNA was omitted and the original 5′-triphosphate on RNA was retained. A close relative, modified bandaid, contains instead 5′ monophosphoryl RNA.

Gels. Electrophoresis was carried out in 0.7% agarose gels with Tris/borate/EDTA running buffer (20). Gels were dried and exposed to XAR-5 film at −70°C. All substrates and components were evaluated electrophoretically using (+)-strand φX174 DNA, linear RNA, and double-stranded linear DNA as markers. The position of bandaid was established by observing a shift in the migration of labeled RNA to the vicinity of innertube DNA upon the addition of single-stranded Bluescript DNA containing complementary sequences. The new band was not affected by quality restriction enzymes, was resistant to RNase A, and, when treated with DNase I, gave rise to the original RNA. Ligated and unligated hybrids contained a new band of slower mobility which, when treated with *Pvu* I or *Pvu* II, was cleaved into labeled fragments of 1.7 and 1.2 kilobases, respectively. These sizes are consistent with a double-stranded Bluescript(KS) 770-like molecule with radioactivity limited to the vicinity of the major histocompatibility complex insert.

RESULTS

Effect of Modified mRNA Termini on the RNase H Activity of AMV Reverse Transcriptase. The products of the RNase H activities of AMV or Moloney murine leukemia virus are oligoribonucleotides 4–30 bases long bearing 5′-phosphates and 3′-hydroxyl groups (for review, see refs. 21 and 22). Therefore, test substrates with different end groups were constructed and evaluated. Globin mRNA was chosen as the RNA moiety for modification because it contains a blocked 5′ terminus, namely, the cap structure; the cap is believed to protect the terminus from attack by cellular exoribonucleases (23). The 3′ end was blocked either by the covalent attachment of pCp or by ligation of a DNA 17-mer, $p(dT)_{17}$, to the poly(A) tail. In each case, RNase H activity was assessed by measuring the rate of deadenylation of globin mRNA in the presence of $oligo(dT)_{12-18}$, where appropriate (13, 22).

As shown in Fig. 2*A*, the rate of cleavage of the poly(A) tails of globin mRNA-pCp in the presence of $oligo(dT)_{12-18}$, was identical to that using globin mRNA under the same conditions. Fig. 2*B* shows that a single curve described the rates of deadenylation of globin mRNA-$p(dT)_{17}$ and globin mRNA mixed with oligo(dT). Initial rates of deadenylation of these substrates were also unchanged by the addition of deoxyribonucleoside triphosphates; the presence of triphosphates makes possible concomitant polymerization of cDNA by the polymerase activity of reverse transcriptase. The data indicate that the T-tail, whether extended or not by the polymerase reaction, had no effect on AMV RNase H activity early in the reaction. (Later, the reaction with mRNA-$p(dT)_{17}$ and triphosphates became complex, as described in the legend to Fig. 2.) In separate experiments, we demonstrated that the cDNA synthesized with mRNA-$p(dT)_{17}$ and with mRNA mixed with oligo(dT) was equivalent (data not shown).

The rates of deadenylation of DNA-terminated globin mRNA in the presence or absence of added $oligo(dT)_{12-18}$ were compared and found to be identical (Fig. 2*B Inset*).

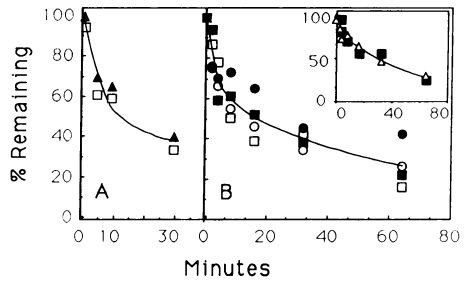

FIG. 2. Effect of 3′-end modifications of globin mRNA-[^{32}P]pA on the rate of deadenylation by AMV reverse transcriptase-associated RNase H. Natural and modified globin mRNAs (2.5 pmol) (specific activity, 3–8 × 10^4 dpm/pmol) were incubated at 37°C in 10 μl containing 10 units of AMV reverse transcriptase, 0.1 μg of $p(dT)_{12-18}$ primer, except where noted, and 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, and 0.5 mM TTP, only where specified, in buffer composed of 50 mM Tris·HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and bovine serum albumin at 50 μg/ml. Points represent normalized Cl_3CCOOH-precipitable radioactivity in 1-μl aliquots (24) at the stated time. (*A*) □, mRNA; ▲, mRNA-pCp. (*B*) □, mRNA; ■, mRNA covalently attached to $p(dT)_{17}$ in the absence of the free primer; ○, mRNA with triphosphates; ●, mRNA-$p(dT)_{17}$ without primer, but with triphosphates. (*Inset*) ■, Defined in *B*; △, mRNA-$p(dT)_{17}$ in the presence of free primer. Due to contaminating rRNA, the end point of deadenylation is ≈30%. [Note: Cleavage of mRNA-[^{32}P]pAp$(dT)_{17}$ by RNase H gives rise to free mRNA with a partial poly(A) tail and an oligomer able to act as a primer for cDNA synthesis on that truncated mRNA, namely, A_n-[^{32}P]pAp$(dT)_{17}$. Thus, tracer that initially becomes acid-soluble as a result of RNase H activity can become and remain acid-insoluble as a consequence of polymerization.]

These data suggest that the T-tail forms a hairpin with poly(A) and that the self-hybrid is the species attacked by the enzyme. If the substrate for RNase H activity had been two uncrimped T-tailed mRNA molecules, each providing oligo(dT) sequences for hybridization with the poly(A) tail of the other, the addition of oligo(dT) should have increased the rate of deadenylation by increasing the number of collisions between poly(A) and oligo(dT).

Results of this study of modified globin mRNA can be interpreted as follows: either the exoribonuclease H activity of AMV reverse transcriptase can recognize a 3′-phosphate or a 3′-hydroxyl group on a DNA tail, or the enzyme is able to attack the hybrid by recognizing the 5′ terminus of globin mRNA without regard for the cap structure. The data demonstrate that the enzyme has extraordinary breadth of specificity or, alternatively, that it is an endoribonuclease.

Characterization of Circular Substrates. Since modified termini were not necessarily blocked with respect to RNase H activity, substrates without termini were required to gain further insight into the catalytic process. Toward this end, a covalently closed circular plasmid with 770 bases of labeled RNA in one strand was synthesized as described in Fig. 1. A preliminary comparison of the ligated hybrids with unligated hybrids revealed that both preparations were similar (Fig. 3); they contained four major radioactive species when examined by gel electrophoresis, which from top to bottom were as follows: base-paired, circular hybrids of various kinds; modified bandaid (see legend to Fig. 1); double-stranded RNA; and single-stranded RNA. (A fifth band was not characterized.) Since ligated and unligated hybrids were virtually indistinguishable under these conditions, purification of ligated hybrids was essential.

Molecules with termini were removed by treatment of the mixture with endonuclease-free Pol I, which expresses both 5′- and 3′-exonucleolytic activity in the absence of triphosphates (Fig. 3). When unligated hybrids were subjected to catalysis, the enzyme destroyed virtually all of the substrate by preferentially, but not exclusively, attacking the DNA component, but when ligated hybrids were used, nicked molecules increased in mobility leaving 30–40% of the total substrate in the original position in the gel. The survivors constitute the covalently closed members of the population and, as an impurity, double-stranded RNA, which is resistant to Pol I. The yield of ligated hybrids was confirmed by digesting the preparation with restriction endonucleases and analyzing the products electrophoretically in denaturing gels (data not shown).

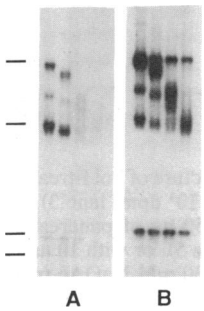

FIG. 3. Treatment of unligated and ligated hybrids with DNA Pol I. Incubation in 20 μl of 0.15 μg of hybrid, 20 units of RNasin, and 7.5 units of *E. coli* Pol I at 37°C in buffer composed of 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, and 1 mM dithiothreitol was used to characterize unligated (*A*) and ligated (*B*) hybrids. Each set displays a time course (from left to right) representing samples withdrawn at 0, 30, 60, and 120 min of treatment and analyzed electrophoretically. Tick marks from *top* to *bottom* indicate the positions of ligated or unligated hybrids, modified bandaid, double-stranded RNA, and single-stranded RNA.

The nature of the ligated hybrids was further investigated (Fig. 4) by incubation with DNase I (lane 2), with RNase A (lane 5), or with mung bean nuclease (lane 4). In each case the expected product was obtained. DNase treatment of the Pol I-treated gel-purified ligated hybrids (lane 1), several days after synthesis, released intact RNA from may of the molecules; failure to recover full-length RNA quantitatively was caused by radioautolysis. Pol I-treated ligated hybrids (lane 3) were completely resistant to RNase and partly resistant to mung bean nuclease, suggesting that the substrate was fully base-paired. That some degradation of these relaxed, circular molecules occurred with a high concentration of the mung bean enzyme was anticipated, based on its known gene-excision activity (25). In contrast, bandaid (lane 6) was reduced to a 770-base-pair heteroduplex when treated with mung bean nuclease under the same conditions (lane 7). Thus, by each of the enzymatic tests devised, the ligated molecules are completely base-paired covalently closed circular DNA·RNA hybrids.

Degradation of Ligated and Unligated Hybrids by Reverse Transcriptases. Circular ligated and unligated DNA·RNA hybrids were tested as substrates for several RNase H activities. Fig. 5 *A–C* displays the time course of degradation of Pol I-treated ligated hybrids by *E. coli*, AMV, and HIV RNase H, respectively. The three RNase H activities almost totally degraded the ligated hybrids. It is significant that a vast majority of the substrate was destroyed; even if perfect ligated hybrids are only a fraction of the total at the time the experiment is performed, they, as well as defective molecules, disappear. That damaged apparently radioautolyzed molecules accumulated in the preparation used in Fig. 5 *A* and *B*, but not yet in *C*, was demonstrated by re-incubation of an aliquot of the ligated hybrids with Pol I (Fig. 5, lanes P). Fig. 5 also demonstrates that digestion of the hybrids resulted in minor changes in mobility. This observation is consistent with the activity of an enzyme that is able to hydrolyze only 770 residues of one strand of a circular molecule consisting of 3700 base pairs.

The unligated hybrids were also tested as substrates for the same three enzymes (Fig. 5 *D–F*). The data show that, for each enzyme, the rate of disappearance of both ligated and unligated hybrids was similar. Therefore, it is unlikely that a contaminant in the reactions presented in Fig. 5 *A–C* converted the ligated hybrids into a form acceptable to putative exonucleases. Specifically, the viral enzymes appear to be unaffected by termini. The data prove that the RNase H activities of HIV and AMV reverse transcriptases are endonucleases.

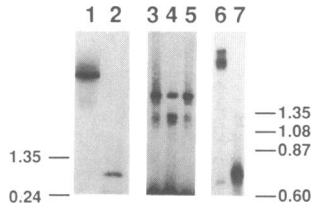

FIG. 4. Proof of structure of Pol I-treated ligated hybrids. Ligated hybrids (0.075 μg, 3 × 10^4 dpm, lane 3) were incubated in 10 μl at 37°C for 60 min with 50 ng of pancreatic RNase A in the buffer described in Fig. 2 (lane 5), or with 10 units of mung bean nuclease in buffer composed of 30 mM NaOAc (pH 5.0), 10 mM $MgCl_2$, 0.1 M NaCl, and 2 mM $ZnCl_2$ (lane 4). As a control, 0.075 μg (10^6 dpm) of bandaid was incubated under the same conditions in the presence (lane 7) or absence (lane 6) of the mung bean enzyme. Ligated hybrids (≈0.3 μg), which were gel-purified in, and excised from, low-melting-temperature agarose containing Tris acetate/EDTA buffer (20) were incubated at 37°C for 30 min in 60 μl of buffer composed of 0.1 M Tris acetate (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol in the presence (lane 2) or absence (lane 1) of 2 units of DNase I. Samples grouped together were in the same gel. Markers, in units of kilobases, to the left are single-stranded RNA (BRL) and refer to lanes 1 and 2; markers to the right are double-stranded DNA and refer to lanes 6 and 7.

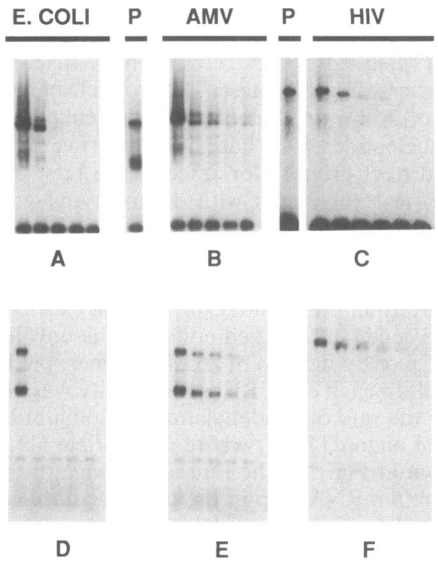

FIG. 5. Effect of HIV, AMV, and *E. coli* RNase H activities on nicked and covalently closed circular DNA·RNA hybrids. Cleavage of 0.15 μg of Pol I-treated ligated hybrids (*A–C*) or unligated hybrids (*D–F*) was measured at 37°C in 20 μl containing 20 units of RNasin with the following enzymes: in *A* and *D*, 0.1 unit of *E. coli* RNase H; in *B* and *E*, 30 (polymerase) units of AMV reverse transcriptase; in *C* and *F*, 2.8 μg of recombinant HIV reverse transcriptase. A time course is shown from left to right with samples withdrawn at 0, 5, 10, 20, and 40 min, and, for the HIV enzyme, also at 80 min. Buffers used in *A*, *B*, *D*, and *E* are described in Fig. 2; those in *C* and *F* are in Fig. 3. The preparations of ligated hybrids in *A* and *B* and unligated hybrids in *D* and *E* were different from those in *C* and *F*, respectively. The lanes P demonstrate the effect of Pol I on the two ligated hybrid preparations in reactions performed concurrently with, and for the same duration as, the test reactions.

Effect of RNase Inhibitors on the RNase H Activity of Reverse Transcriptases. The effect of two inhibitors of RNase, ribonucleoside-vanadyl complexes (26) and RNasin, on the RNase H activity of reverse transcriptase from AMV and HIV was determined. As shown in Fig. 6, the two RNase H activities were unchanged in the presence of RNasin. In contrast, vanadyl complexes inhibited both enzymes. Although the mechanism of the inhibition with vanadyl complexes is unknown in this case, the effect is significant because vanadyl complexes do not inhibit the commercially available *E. coli* RNase H (13).

DISCUSSION

The reverse transcriptase-associated RNase H activities of AMV (10, 11), Moloney murine leukemia virus (27, 28),

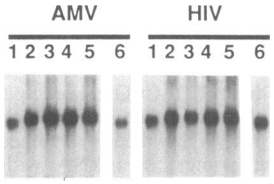

FIG. 6. Effect of RNase inhibitors on RNase H activity. The effect of 0, 2.5, 5, 10, and 20 mM ribonucleoside-vanadyl complexes (lanes 1–5, respectively) or 10 units of RNasin (lane 6) on the catalytic activity of 1.4 μg of HIV or 15 (polymerase) units of AMV reverse transcriptase was measured at 37°C for 30 min in 10 μl containing 0.075 μg (6 × 10^5 dpm) of bandaid in buffers specified for each enzyme in Fig. 5. In lanes 1, 53% (AMV) and 52% (HIV) of the substrate remain; the values for lanes 6 are 48% and 45%, respectively.

Friend murine leukemia virus (29), and HIV (30) were believed to be exonucleases because they are unable to cleave certain circular DNA·RNA hybrids that serve as substrates for *E. coli* RNase H. Nevertheless, based on the intermediates encountered in the conversion of viral genomic RNA into double-stranded DNA *in vivo*, virologists began to treat these enzymes conceptually as endonucleases. The complexity of the RNase H substrates found in retrovirus-infected cells was responsible. DNA·DNA, RNA·RNA, and RNA·DNA hybrids coexisted as parts of gapped circular molecules, with many bases and extended regions of secondary structure sometimes separating the target sequences for RNase H from the nearest 5′-phosphoryl or 3′-hydroxyl RNA terminus (reviewed in refs. 21 and 31). In particular, it was difficult to envisage how an exoribonuclease H could remove the tRNA that acts as a primer for minus-strand DNA synthesis. The structure involved consists of a tRNA molecule that is covalently linked at its 3′ end to minus-strand DNA, and hybridized, in part, to plus-strand DNA. Specifically, the last ≈20 bases at the 3′ end of the tRNA, as well as the attached minus-strand DNA, are base-paired to plus-strand DNA residues (4). To cleave this substrate, an exonuclease would have to recognize either a DNA terminus or the 5′-phosphoryl terminus of the tRNA; the latter is separated from the tRNA·DNA hybridized region by ≈60 bases of folded tRNA.

Our experiments with linear substrates containing end-modifications suggested that, if the RNase H activity of AMV reverse transcriptase were indeed an exonuclease, a wide variety of termini were acceptable. Furthermore, Gerard (27), using analogs of the natural tRNA-containing substrate composed of poly(dT) hybridized either to $C_{\approx 30}A_n(dA)_{\approx 60}$ or to $C_{\approx 60}A_n(dA)_{\approx 140}$, was able to demonstrate release of oligo(A) from the hybrid with Moloney murine leukemia virus reverse transcriptase. He concluded that an exoribonuclease H could indeed accommodate the natural substrate.

To resolve the issue, it was necessary to determine whether a circular molecule containing a DNA·RNA hybrid could serve as a substrate for viral reverse transcriptase-associated RNase H activities. In designing a test molecule, we wished to avoid the pitfalls of earlier constructs. For example, poly(A) circles rarely exceed 15 or 16 residues (32); the ability to circularize RNA is strictly dependent on the size of the homopolymer with markedly reduced yields at higher degrees of polymerization. Similarly, the number of consecutive ribonucleotides in supercoiled plasmid DNA was questionable. ColE1 DNA plasmids synthesized in the presence of chloramphenicol contain short stretches of RNA which are unlikely to exceed the length of the RNA primer, about 25 residues (28). Clearly, if the conformation of the DNA·RNA hybrid were to play no role in the experimental outcome, the RNA moiety must be both large and varied in base composition. Therefore, we constructed a perfectly base-paired relaxed circular molecule, without nicks, in which the DNA·RNA region could assume a structure identical to that of linear substrates. With 770 consecutive ribonucleotides, at least part of the DNA·RNA hybrid region should be in the "correct" form, despite the effect of large DNA·DNA hybrids flanking both ends of the RNA. Thus, we attempted to eliminate termini without also affecting the conformation of the hybrid.

Our results with the circular substrate, ligated hybrid, are unambiguous in showing that the RNase H activities of AMV and HIV are endonucleases. There is no indication that viral enzymes recognize or are affected by termini. Hence, molecules modeled on termini are not likely to be specific inhibitors. Nevertheless, the data suggest differences in mechanism between cellular RNase H activities, as exemplified by the commercially available enzyme from *E. coli* and several mammalian RNase H activities, and the viral enzymes. One can infer that the viral enzymes are more sensitive to the secondary structure of the DNA·RNA hybrid or that they require longer hybrids in the correct conformation than the normal enzymes. This condition might reflect a difference in the active sites of the enzymes, with the normal enzymes better able to mold themselves around a distorted or strained substrate than the viral enzymes. Such a dissimilarity could be exploited for drug design; the viral enzymes appear to be more fastidious than the cellular enzymes and, as a consequence, they might be more easily inhibited. The fact that ribonucleoside-vanadyl complexes inhibit viral RNase H, but not the *E. coli* enzyme (13), would tend to support this view.

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