Biochemistry

 Volume 30, Number 29 July 23, 1991

Accelerated Publications

Human Immunodeficiency Virus Reverse Transcriptase Ribonuclease H: Specificity of tRNA^{Lys3}-Primer Excision†

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Received April 19, 1991; Revised Manuscript Received May 28, 1991

ABSTRACT: Two model substrates were prepared to examine the mechanism of tRNA-primer excision catalyzed by reverse transcriptase associated ribonuclease H (RT-RNase H). The first model substrate contained sequences from the HIV genome and was designed to be structurally similar to the DNA-extended tRNA created by initiation of minus-strand DNA synthesis during retroviral replication. The DNA-extended RNA was a template and was annealed to a DNA oligonucleotide that primed reverse transcription of the RNA in the template. The second model substrate was structurally similar the first substrate but contained sequences unrelated to the HIV viral genome. The RT-RNase H catalyzed excision of the RNA from the template of the two model substrates was examined. Human immunodeficiency virus (HIV) and Moloney murine leukemia virus RT-RNase H hydrolyzed the substrates to leave a single ribonucleotide 5′-phosphate at the 5′-terminus of the model DNA genome. In contrast, avian myeloblastosis virus RT-RNase H hydrolyzed the phosphodiester bond at the DNA–RNA junction. These hydrolytic specificities were not highly dependent on substrate sequence. The importance of these specificities to retroviral integration is discussed. Additional data indicated that the HIV polymerase and RNase H active sites are separated by a distance equivalent to the length of a 15-nucleotide RNA–DNA heteroduplex.

The enzyme responsible for replication of the human immunodeficiency virus (HIV)[1] genome, reverse transcriptase (RT), is both a DNA polymerase and a RNase H (Hansen et al., 1987, 1988; Becerra et al., 1990). The native enzyme is a heterodimer composed of a 66- and 51-kDa subunit (Di Marzo-Veronese et al., 1986; Lightfoote et al., 1986). The RNase H active site is, at least in part, in the carboxyl-terminal domain of the 66-kDa polypeptide (Hansen et al., 1987, 1988; Davies et al., 1991).

Reverse transcription of retroviral genomes requires a specific tRNA (tRNA^{Lys3} in the case of HIV) to prime first-strand ("minus-strand") DNA synthesis (Giboa et al., 1979). RNase H has at least three roles subsequent to initiation of reverse transcription. First, the enzyme partially degrades the RNA template during/after first-strand synthesis, leaving a specific RNA primer ("polypurine tract") for initiation of second-strand ("plus-strand") DNA synthesis (Mitra et al., 1979, 1982; Finston & Champoux, 1984; Rattray & Champoux, 1987; Resnick et al., 1984). Second, RNase H excises the polypurine tract RNA primer from plus-strand DNA by hydrolysis of the phosphodiester bond at the RNA–DNA junction (Champoux et al., 1984; Huber & Richardson, 1990). Finally, the tRNA primer is removed from the minus-strand DNA (Gilboa et al., 1979; Omer & Faras, 1982). Excision of the tRNA forms the 5′-terminus of the minus strand of the linear double-stranded DNA copy of the viral genome (Gilboa et al., 1979). Avian sarcoma virus (ASV) RT-RNase H excises the intact tRNA primer (Omer & Faras, 1982). In the present study, the specificity of HIV RT-RNase

† This paper is dedicated to Professor Robert H. Abeles in honor of his 65th birthday. His contributions to biochemistry as a scientist and educator have enriched us all.

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[1] Abbreviations: HIV, human immunodeficiency virus; MMuLV, Moloney murine leukemia virus; AMV, avian myeloblastosis virus; RT, reverse transcriptase; RT-RNase H, reverse transcriptase associated RNase H; RNAsin, human placental RNase inhibitor; dNTP, 2′-deoxynucleoside 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TBE, Tris–borate–EDTA.

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H catalyzed tRNA-primer excision from two model substrates was investigated. Further, the specificity of HIV RT-RNase H was compared to that of RT-RNase H from AMV and MMuLV.

Materials and Methods

Synthetic DNA oligonucleotides (Midland Certified Reagent Co., Midland, TX) are (1) 5′-tgg cgc ccg aac agg gac cct ata gtg agt cgt att a-3′ (see below) and (2) 5′-taa tac gac tca cta ta-3′ (see below).

Synthesis of RNA (for Template 1). DNA oligonucleotide 1 (7 OD units at 260 nm/mL) and DNA oligonucleotide 2 (3 OD units at 260 nm/mL) were annealed in Tris·HCl (10 mM, pH 8) and EDTA (1 mM) by heating to 90 °C for 2 min and then cooling slowly from 65 °C to room temperature. The resulting oligo-1/oligo-2 duplex (0.32 OD unit at 260 nm/mL) was transcribed with T7 RNA polymerase (BRL, Bethesda, MD; 700 units) as described by the manufacturer. The DNA was digested by the addition of RNase-free DNase (Promega, Madison, WI). The mixture was extracted with phenol/chloroform, and the small molecules were removed by centrifuge-facilitated chromatography on Sephadex G-50 (Maniatis et al., 1980). The effluent mixture was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN; 10 units), Tris·HCl (100 mM, pH 8.0), and $MgCl_2$ (10 mM) for 30 min at 37 °C. The resulting solution was extracted with phenol/chloroform and desalted by Sephadex G-50 chromatography as above. The mixture was electrophoresed through a 20% polyacrylamide/8 M urea gel, and the 20-nucleotide RNA product was visualized by UV shadowing, excised from the gel, and electroeluted from the gel with an Elutrap (Schleicher and Schuell).

Synthesis of RNA (for Template 2). RNA for template 2 (Figure 1) was synthesized by transcribing 25 μg of *Sma*I-hydrolyzed pGEM3zf- (Promega, Madison, WI) with T7 RNA polymerase as described above. The RNA was purified as above.

5′ Radiolabeling of RNA Oligonucleotides. RNA oligonucleotides (0.2 μg) were radiolabeled with [γ-^{32}P]ATP (Du Pont New England Nuclear Products, Wilmington, DE) by using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), as described by the manufacturer. The specific activity of the purified RNA was approximately 10^7 cpm/μg. Concentrations of starting oligonucleotides were determined by 1 absorbance unit at 260 nm = 20 μg/mL.

Synthesis of Template 1 (DNA Labeled). RNA for template 1 (280 ng), primer 1 (Figure 1, 400 ng), Tris·HCl (24 mM, pH 7.4), $MgCl_2$ (6 mM), and KCl (60 mM), in a volume of 80 μL, were heated to 90 °C for 2 min and then cooled slowly from 65 °C to room temperature. Deoxy-NTP's (50 μM final concentration), [α-^{32}P]dGTP (Du Pont New England Nuclear Products, Wilmington, DE; 30 μCi), RNAsin (200 units), and Klenow (Boehringer Mannheim, Indianapolis, IN; 20 units) were added to initiate extension of the RNA primer. The mixture was incubated at 37 °C for 10 min and then quenched by extraction with phenol/chloroform. Small molecules were removed by Sephadex G-50 chromatography. The sample was electrophoresed through a 15% polyacrylamide/8 M urea gel. The extension product was excised as a gel slice and eluted with the Elutrap.

Synthesis of Template 1 (RNA Labeled). RNA-labeled template 1 was prepared as described above for the DNA-labeled strand except that [α-^{32}P]dGTP was omitted and the annealing mixture contained [5′-^{32}P]RNA-1 (10^6 cpm).

Synthesis of Template 2 (DNA and RNA Labeled). These nucleic acids were prepared in a manner analogous to that of template-1 with RNA for template 2 and primer 2 (Figure 1) replacing the RNA for template 1 and primer 1, respectively.

Annealing of Model Template/Primers. All template/primer strands were heated in 100 mM Tris-HCl (pH 8), 100 mM KCl, 4 mM dithiothreitol, and 10 mM $MgCl_2$ at 90 °C for 2 min and then cooled slowly from 65 °C to room temperature. The approximate concentrations of nucleic acids were 0.3 ng/μL for templates and 1.5 ng/μL for primers.

Ribonuclease and Alkaline Digestion of Rabiolabeled Nucleic Acids. The RNA portion of the molecule was sequenced by using alkaline digestion and the guanine-specific ribonuclease T1 (DNA-labeled template/primer 1, Figure 2, lanes 1 and 2; DNA-labeled template/primer 2, Figure 4, lanes 1 and 2). Alkaline and ribonuclease ribonuclease T1 and U2 digestions were performed as described in the RNA sequencing kit (Pharmacia, Piscataway, NJ).

Enzyme Assays. Immunoaffinity-purified HIV reverse transcriptase was kindly provided by Dr. Philip A. Furman, Wellcome Research Laboratories. All assay mixtures contained a single reverse transcriptase [HIV, 3 μg/mL; MMuLV, 10 μg/mL (BRL, Bethesda, MD); AMV, 60 μg/mL (Pharmacia, Piscataway, NJ)], 0.15 μg/μL nuclease-free bovine serum albumin (Pharmacia, Piscataway, NJ), 4 units/μL RNAsin, and 2′-deoxynucleoside 5′-triphosphates (11 μM). Assays were initiated by combining a 2× stock of the components above with an equivalent volume of annealed template/primer at 37 °C. In all cases, aliquots (5 μL) were removed from the reactions at the indicated times and quenched by addition to 5 μL of Maxam–Gilbert loading dye (0.1% bromophenol blue and xylene cyanol; 50 mM Tris–borate, pH 8.3; 1 mM EDTA in 80% formamide). The samples were heated to 90 °C for 2 min, cooled on ice for at least 2 min, and electrophoresed through a polyacrylamide/8 M urea gel.

Preparation and Analysis of Products 1 and 2. Enzyme reactions with HIV RT-RNase H, dNTP's, and either template/primer 1 or template/primer 2 (DNA labeled) were increased to 100-μL total volume. Products were purified by polyacrylamide/urea gel electrophoresis. For dephosphorylations, product 1 or 2 was treated with calf intestinal alkaline phosphatase, as described by the manufacturer.

Results and Discussion

Two model substrates were prepared to investigate the specificity of tRNA-primer removal by the HIV RT-RNase H. Template/primer 1 (Figure 1) contained a 20-nucleotide RNA strand in which the 18 nucleotides of the 3′-terminus were identical with those of $tRNA^{Lys3}$.[2] To the 3′-end of this RNA oligonucleotide was attached a 15-nucleotide DNA strand with a sequence based on a consensus sequence of the first 15 nucleotides produced by reverse transcription of the HIV LTR.[2] This DNA-extended RNA "template" was annealed to a DNA oligonucleotide primer that overlapped the DNA–RNA junction. Since this template/primer was a substrate for reverse transcription, it was an appropriate model to study RT-RNase H catalyzed excision of the $tRNA^{Lys3}$ primer during DNA synthesis. A similar substrate (template/primer 2) was synthesized that contained little sequence homology to $tRNA^{Lys3}$ or the HIV genome. Templates 1 and 2 were labeled with either ^{32}P at the 5′-terminus of the RNA or [α-^{32}P]dGTP in the DNA during DNA synthesis.

[2] The sequences for $tRNA^{Lys3}$ and its DNA extension were selected from consensus sequences of the HIV primer binding-site region (Meyers, 1990).

Template/primer 1

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              5         10        15        20
5'-rGrGrGrUrCrCrCrUrGrUrUrCrGrGrGrCrGrCrCrA*dCdTdGdCdTdAdGdAdGdAdTdTdTdTdT-3'
              3'-dGdAdCdAdAdGdCdCdCdGdCdGdGdT-dGdAdCdGdAdTdCdTdCdTdAdAdAdAdA-5'
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Template/primer 2

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              5         10        15        20
5'-rGrGrGrCrGrArArUrUrCrGrArGrCrUrCrGrGrUrArCrCrC*dGdGdGdGdAdTdCdCdTdCdTdAdG-3'
                        3'-dTdCdGdAdGdCdCdAdTdGdGdG-dCdCdCdCdTdAdGdGdAdGdAdTdC-5'
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FIGURE 1: Model substrates for tRNA excision. The asterisks indicate the phosphodiester bond of the DNA–RNA junction.

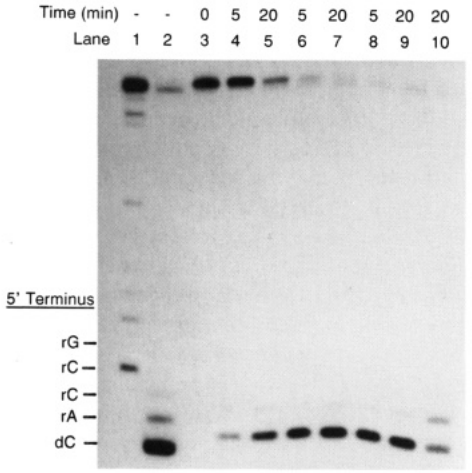

FIGURE 2: Reaction of HIV RT-RNase H with template/primer 1 (DNA labeled): lanes 1 and 2, ribonuclease T1 and alkaline digestion of template 1; lane 3, untreated template/primer 1; lanes 4 and 5, template/primer 1 and RT-RNase H, 5- and 20-min incubation; lanes 6 and 7, template/primer 1, RT-RNase H, and dGTP, 5- and 20-min incubation; lanes 8 and 9, template/primer 1, RT-RNase H, and dGTP, dATP, and dCTP, 5- and 20-min incubation; lane 10, template/primer 1 and *E. coli* RNase H, 20-min incubation. The left legend defines the 5′-terminal nucleotide of the respective band; r = ribo and d = 2′-deoxyribo. The figure is an autoradiogram of a 15% polyacrylamide/8 M urea gel.

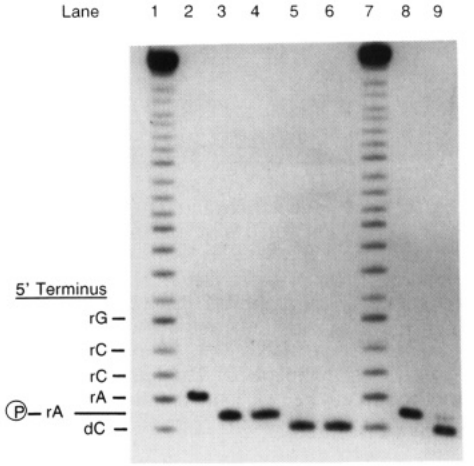

FIGURE 3: Chemical and enzymatic analysis of product 1: lanes 1 and 7, alkaline digestion of template 1; lane 2, alkaline phosphatase treated product 1; lanes 3 and 8, untreated product 1; lanes 4–6, alkaline hydrolysis of product 1, 5-, 15-, and 30-min incubation; lane 9, ribonuclease U2 digestion of product 1. The left legend defines the 5′-terminal nucleotide of the respective band; r = ribo, d = 2′-deoxyribo, and P = phosphate. The figure is an autoradiogram of a 15% polyacrylamide/8 M urea gel.

One major product (product 1) was observed after HIV RT-RNase H was incubated with template/primer 1 (DNA labeled), in the presence or absence of dNTP's (Figure 2, lanes 3–9). Product 1 migrated between the two shortest products of the alkaline hydrolysis reaction (Figure 2: compare lane 2 to lane 4). The shortest nucleic acid fragment in the alkaline digest was the DNA portion of template 1 (DNA-1). The next fragment was DNA-1 with adenosine on the 5′-terminus (5′-rA-DNA-1). Alkaline hydrolysis of RNA leaves a 5′-hydroxyl group while HIV RT-RNase H leaves a 5′-phosphate (Starnes & Cheng, 1989). Since product 1 migrated between 5′-rA-DNA-1 and DNA-1, product 1 was most likely 5′-rA-DNA-1 with a 5′-terminal phosphate (5′-P-rA-DNA-1).

The structure of product 1 was characterized by a series of digestions. An alkaline digest of the template provided standard markers (Figure 3, lanes 1 and 7). Treatment of product 1 with alkaline phosphatase produced an oligonucleotide that comigrated with the 5′-rA-DNA-1 fragment in the alkaline digest (Figure 3, lane 2). This result was consistent with removal of a phosphate from 5′-P-rA-DNA-1. Alkaline digestion of product 1 produced a product that comigrated with DNA-1, consistent with the removal of adenosine 3′,5′-diphosphate (Figure 3, lanes 4–6). Similarly, if product 1 was treated with ribonuclease U2 (an adenosine-specific ribonuclease), the new product comigrated with DNA-1 (Figure 3, lane 9). These data demonstrated that product 1 was 5′-P-rA-DNA-1. Thus, RT-RNase H catalyzed the hydrolysis of the phosphodiester bond between rC19 and rA20 (Figure 1), and not at the DNA–RNA junction.

The products of the HIV RT-RNase H catalyzed hydrolysis of template/primer 1 (RNA labeled) were examined as described for the DNA-labeled template/primer 1. The primary product was the RNA portion of template 1 without the 3′-terminal adenosine 5′-phosphate (data not shown). This result suggests, in vivo, the excised tRNA primer does not contain the 3′-terminal adenosine 5′-phosphate but is otherwise intact.

Template/primer 2 was examined to determine whether the specificity of the HIV RT-RNase H hydrolysis of the phosphodiester bond between rC19 and rA20 of template/primer 1 was the result of an inherent specificity for the nucleotide sequence of the HIV primer binding site. HIV RT-RNase H was incubated with template/primer 2 (DNA labeled) in the presence or absence of dNTP's (Figure 4). Two products were formed from the RNase H catalyzed hydrolysis of template/primer 2 in the presence of dCTP or in the absence of nucleotides. The shorter of these two hydrolysis products corresponded to DNA-2 with four ribonucleotides at the 5′-terminus, and the longer of these two products corresponded to DNA-2 with five ribonucleotides at the 5′-terminus. With a mixture of dCTP, dGTP, and dATP, one major product (product 2) was observed (Figure 4, lanes 8 and 9). Product 2 was characterized by the methods used with product 1 (data not shown) and identified as DNA-2 with a 5′-terminal cytosine 5′-phosphate (5′-P-rC-DNA-2). Thus, HIV RT-RNase

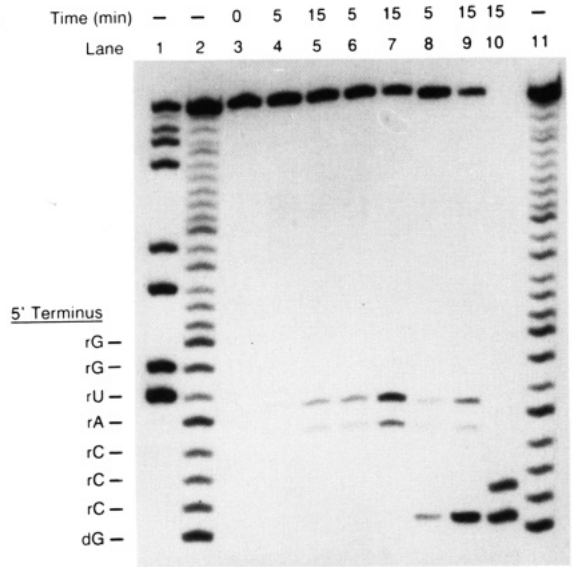

FIGURE 4: Reaction of HIV RT-RNase H with template/primer 2 (DNA labeled): lanes 1 and 2, ribonuclease T1 and alkaline digestion of template 2; lane 3, untreated template/primer 2; lanes 4 and 5, template/primer 2 and RT-RNase H, 5- and 15-min incubation; lanes 6 and 7, template/primer 2, RT-RNase H, and dCTP, 5- and 15-min incubation; lanes 8 and 9, template/primer 2, RT-RNase H, and dGTP, dATP, and dCTP, 5- and 15-min incubation; lane 10, template/primer 2 and *E. coli* RNase H, 15-min incubation; lane 11, alkaline hydrolysis of template 2. The left legend defines the 5′-terminal nucleotide of the respective band; r = ribo and d = 2′-deoxyribo. The figure is an autoradiogram of a 20% polyacrylamide/8 M urea gel.

H hydrolyzed the phosphodiester bond between rC22 and rC23 (Figure 1), and not the bond at the RNA–DNA junction. Further, the specificity for hydrolysis of a bond one nucleotide removed from the bond at the DNA–RNA junction was not determined by template/primer sequence.

The RNA sequences of template/primers 1 and 2 are similar near the DNA–RNA junction (1 = rCrCrA and 2 = rCrCrC), though there is very low overall homology in the RNA sequences. A third template/primer was examined as a model substrate to eliminate the possibility that the specificity for the bond one nucleotide removed from the bond at the DNA–RNA junction was due to this homology near the junction. The RNA sequence at the DNA–RNA junction was rCrGrG.[3] The major product from HIV RT-RNase H hydrolysis of this template/primer was the DNA of the template with a 5′-terminal guanosine 5′-phosphate. This result indicated that the primary cleavage site was between the two guanine nucleotides closest to the DNA–RNA junction (data not shown). Once again, HIV RT-RNase H hydrolyzed the RNA phosphodiester bond that was one bond removed from the bond at the DNA–RNA junction.

To show that these template/primers were normal substrates for any RNase H and not dependent upon RT-associated RNase H, *Escherichia coli* RNase H (which does not contain RT activity) was reacted with template/primer 1 (Figure 2, lane 10). Product 1 and a second product (DNA-1 with two ribonucleotide 5′-phosphates at the 5′-terminus) were formed. Analogous results were obtained with template/primer 2 (Figure 4, lane 10). These results demonstrate that *E. coli* RNase H cleaved the phosphodiester bond one nucleotide removed from the DNA–RNA junction, but not at the DNA–RNA junction. However, a substantial portion of the reaction products included DNA strands with two ribonucleotide 5′-phosphates at the 5′-terminus. Further, examination of the *E. coli* RNase H hydrolysis of RNA-labeled template/primer 1 demonstrated several prominent cleavages throughout the region of RNA in the heteroduplex (data not shown). Thus, the *E. coli* enzyme did not show specificity for a single bond, in contrast to the results observed with HIV RT-RNase H.

The RNA hydrolytic activity of HIV RT-RNase H has been categorized into two classes. The classes include a "polymerase-independent" and a "polymerase-dependent" RNA hydrolytic activity (Furfine & Reardon, 1991). In the polymerase-independent binding mode, RT-RNase H can catalyze RNA hydrolysis at many sites in the RNA up to six nucleotides from the ribonucleotide complementary to the 3′-terminal deoxynucleotide of the primer. The polymerase-independent RNase H activity is not coupled to polymerase catalysis; however, it may be sequence dependent. The two sites of hydrolysis observed with template/primer 2 in the absence of nucleotides or in the presence of dCTP (Figure 4, lanes 4–7) were considered to be polymerase-independent cleavages. The products of these two cleavages were DNA-2 with 4 and 5 ribonucleotide 5′-phosphates, respectively, at the 5′-terminus. In the polymerase-dependent binding mode, the specificity of HIV RT-RNase H is defined, in part, by binding of the polymerase active site to the 3′-terminus of the primer. In this binding mode, the RNase H active site is positioned 15–16 nucleotides upstream of the primer 3′-terminus. In the present study, the rate of RNA hydrolysis of template/primer 1 (DNA labeled) at the phosphodiester bond between rC19 and rA20 was dramatically increased when dGTP was included in the reaction mixture (Figure 2: compare lanes 4 and 5 to lanes 6 and 7). The template RNA encodes the incorporation of two dGMP residues into primer 1. Extension of primer 1 by two nucleotides moves the phosphodiester bond between rC19 and rA20 from a distance of 13 nucleotides to a distance of 15 nucleotides upstream of the primer 3′-terminus. In the polymerase-dependent binding mode, the RNase H active site need only be positioned at a site transiently during reverse transcription to enhance the hydrolysis at the site. For example, the hydrolysis between rC19 and rC20 is enhanced even when primer 1 encoded extension by more than two nucleotides (six nucleotides in the presence of dGTP, dATP, and dCTP) (Figure 2: compare lanes 4 and 5 to lanes 8 and 9). Further, HIV RT-RNase H did not not hydrolyze the phosphodiester bond of template/primer 2 between rC22 and rC23 unless dCTP, dGTP, and dATP were included in the reaction mixture (Figure 4: compare lanes 4–7 to lanes 8 and 9). In the presence of dCTP, dGTP, and dATP, the template encodes the extension of primer 2 by four nucleotides. Extension of primer 2 by four nucleotides positions the bond between rC22 and rC23 15 nucleotides upstream from the 3′-terminus. Thus, these results are consistent with a model for RT-RNase H in which the distance between the polymerase and RNase H active sites is equivalent to the length of a 15-nucleotide RNA–DNA heteroduplex. With both template/primers, hydrolysis of the bond at the DNA–RNA junction was not observed even when the RNase H active site was positioned there transiently during reverse transcription.

The consensus sequence of the HIV primer binding site[2] is complementary to at least the first 18 nucleotides from the 3′-terminus of $tRNA^{Lys3}$. Therefore, the reverse transcriptase most likely reverse transcribes the tRNA at least 18 nucleotides

[3] The complete template sequence is 5′-rGrGrGrCrGrArArUrUrCrGrArGrCrUrCrGrGrUrArCrCrCrGrG*-dGdGdAdTdCdCdTdCdTdAdG-3′. This template was annealed with primer 2 to make the third model substrate.

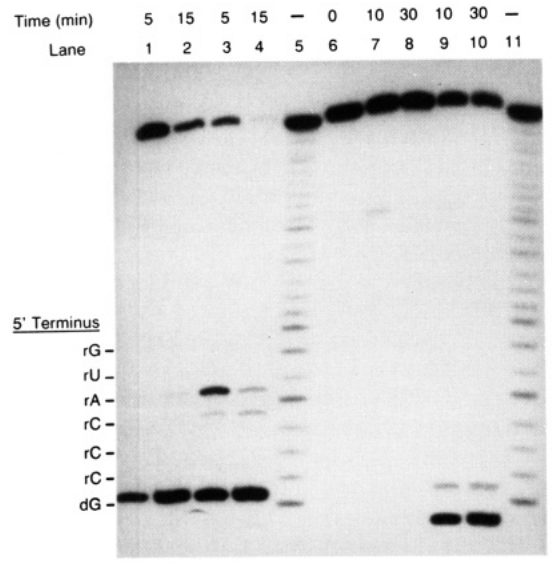

FIGURE 5: Reaction of RT-RNase H from MMuLV and AMV with template/primer 2 (DNA labeled): lanes 1 and 2, template/primer 2 and MMuLV RT-RNase H, 5- and 15-min incubation; lanes 3 and 4, template/primer 2, MMuLV RT-RNase H, and dGTP, dATP, and dCTP, 5- and 15-min incubation; lanes 5 and 11, alkaline digestion of template 2; lane 6, untreated template/primer 2; lanes 7 and 8, template/primer 2 and AMV RT-RNase H, 10- and 30-min incubation; lanes 9 and 10, template/primer 2, AMV RT-RNase H, and dGTP, dATP, and dCTP, 5- and 20-min incubation. The left legend defines the 5′-terminal nucleotide of the respective band; r = ribo and d = 2′-deoxyribo. The figure is an autoradiogram of a 20% polyacrylamide/8 M urea gel.

past the DNA–RNA junction. This reverse transcription of the tRNA is an integral step in retroviral genome replication (Gilboa et al., 1979). Since the distance between the polymerase and RNase H active sites is equal to a 15-nucleotide DNA–RNA heteroduplex, and transcription of at least 18 nucleotides of the tRNA occurs, the excision of the tRNA can occur in the polymerase-dependent binding mode. Further, if tRNA excision is polymerase dependent, it can occur during reverse transcription, not subsequent to completion of DNA synthesis.

The results of MMuLV RT-RNase H hydrolysis of template/primers 1 and 2 (DNA labeled) were similar to the results obtained with HIV RT-RNase H (template/primer 2, Figure 5, lanes 1–4; template/primer 1, data not shown). The MMuLV enzyme hydrolyzed the phosphodiester bond between rC19 and rA20 (template/primer 1) and betwen rC22 and rC23 (template/primer 2) but not the phosphodiester bond of the DNA–RNA junctions. Interestingly, AMV RT-RNase H had a markedly different specificity. In the absence of nucleotides, hydrolysis was negligible (Figure 5, lanes 7 and 8). A small amount of product was observed (DNA-2 with 15 ribonucleotide 5′-phosphates at the 5′-terminus). In the presence of dCTP, dGTP, and dATP, AMV RT-RNase H hydrolyzed template/primer 2 to give a product that migrated farther than DNA-2 (Figure 5, lanes 9 and 10). As discussed previously, the products of the RT-RNase H catalyzed hydrolysis of a phosphodiester bond are a 3′-hydroxyl group and a 5′-phosphate (Starnes & Cheng, 1989). Therefore, it appeared that the product was the DNA of template 2 with a 5′-terminal phosphate (5′-P-DNA-2). Consistent with this analysis, dephosphorylation of the product with alkaline phosphatase produced an oligonucleotide that comigrated with DNA-2 (data not shown). The analogous product (5′-P-DNA-1) was formed when AMV RT-RNase H hydrolyzed template/primer 1 (data not shown). These data indicated that AMV RT-RNase H hydrolyzed the phosphodiester bond at the DNA–RNA junction. Thus, all RT-RNase H's did not have the same specificity for tRNA primer excision. Further, in all cases, the specificities of RT-RNase H's were distinct from that of *E. coli* RNase H.[4]

HIV and MMuLV RT-RNase H exhibited the same specificity for hydrolysis of template/primers 1 and 2. However, the relative amounts of the products formed differed. In addition, the specificity of the two enzymes differed in their dependence on dNTP's. For example, both enzymes hydrolyzed the bond one nucleotide removed from the DNA–RNA junction of template/primer 2. However, MMuLV RT-RNase H catalyzed this hydrolysis in the presence and absence of dNTP's, whereas HIV RT-RNase H catalyzed this hydrolysis only in the presence of dNTP's. Further, both enzymes hydrolyzed template/primer 2 at sites 4 and 5 nucleotides from the DNA–RNA junction, although the dependence of these cleavages on the presence of nucleotides differed as well. These differences in RNA hydrolytic specificity may be, in part, due to differences in the spatial relationship of the polymerase and RNase H active sites in these enzymes. The relationships between the RNase H and polymerase active sites of MMuLV and AMV RT-RNase H are not well-defined, although an interactive site distance for the MMuLV enzyme of a 7–14 nucleotide DNA–RNA heteroduplex has been reported (Oyama et al., 1989).

The products of the tRNA-primer excision reaction depend upon the viral origin of the RT-RNase H that catalyzes the reaction. These hydrolytic specificities may be related to the respective mechanisms of viral integration. Junctions of circularized double-stranded DNA forms of the HIV, MMuLV, and AMV retroviral genomes have been sequenced. Though the circular DNA is not thought to be a replicative intermediate, the junctions of these circular DNA species are most likely representative of the termini from the linear preintegrative form of the viral genome (Lobel et al. 1989). In the case of HIV, the sequence of the circle junction suggests that the 5′-terminus of the minus strand of the linear double-stranded DNA copy of the viral genome contains an "extra" adenine nucleotide (Smith et al., 1990; Whitcomb et al., 1990; Pullen & Champoux, 1990; Kulkosky et al., 1990). This nucleotide could have been derived from the 3′-terminus of the $tRNA^{Lys3}$ primer. The results in the present study have demonstrated that HIV RT-RNase H hydrolyzed the model template/primers to produce a DNA product containing a single 5′-terminal adenine nucleotide. This result is consistent with the extra nucleotide in the junction being derived from the tRNA primer. In contrast, the AMV circle junction sequence does not contain extra nucleotides, suggesting that AMV RT-RNase H hydrolyzes the bond of the DNA–RNA junction to create the terminus of the linear viral genome (Grandgenett et al., 1986). Accordingly, the data in this report and the data in a previous report (Omer & Faras, 1982) deomonstrate that both AMV and ASV RT-RNase H [an enzyme closely related to AMV RT-RNase H (Verma, 1977)] hydrolyzed the DNA–RNA junction during tRNA excision. Wild-type circle junction sequences from MMuLV genomes

[4] The commercially available MMuLV RT-RNase H, prepared by cloning in *E. coli*, was devoid of any contaminating *E. coli* RNase H (compare Figure 5, lanes 1–4, to Figure 4, lane 10). Similarly, immunoaffinity-purified HIV RT-RNase H cloned in *E. coli* was devoid of any contaminating *E. coli* RNase H activity (Furfine & Reardon, 1991). Finally, commercial AMV RT-RNase H, highly purified from infected chicks, depended upon the presence of nucleotides for activity (Figure 5, lanes 7–10). Since a chick cell RNase H would not likely be associated with a reverse transcriptase, it was unlikely that AMV RT-RNase H was contaminated with chick cell derived RNase H.

also indicate that RT-RNase H hydrolyzes the DNA–RNA junction (Shoemaker et al., 1980). These findings are inconsistent with our data demonstrating that MMuLV RT-RNase H hydrolyzed model template/primers to produce a DNA product containing a single 5′-terminal ribonucleotide. However, circle junction sequences from mutant MMuLV genomes indicate that the RNase H can leave one or more ribonucleotides at the terminus of the linear viral genome (Colicelli & Goff, 1988).

The products of HIV RT-RNase H hydrolysis of the model template/primers reported here and the sequence of the HIV circle junction suggest that the natural linear DNA substrate for the HIV integration protein contains a 5′-terminal adenosine 5′-phosphate moiety. Consistent with this, the HIV integration protein efficiently utilized a double-stranded DNA substrate containing a 5′-terminal adenosine 5′-phosphate in an in vitro integration/cleavage reaction (Paula Sherman, personal communication). Further, if RT-RNase H were to hydrolyze the DNA–RNA junction during tRNA-primer excision, the linear genome would be one nucleotide shorter. Model substrates for the HIV integration protein, which are one nucleotide shorter than the wild-type model substrates, are 5–10-fold less efficient substrates compared to the wild type (Paula Sherman, personal communication). Thus, the RT-RNase H hydrolysis of the RNA–RNA bond one nucleotide removed from the DNA–RNA junction may be critical to the efficient integration of HIV linear DNA genomes. Finally, the specificity of HIV RT–RNase H reported here for hydrolysis of template/primers that model tRNA-primer excision may accurately reflect the specificity of RT-RNase H in vivo.

ACKNOWLEDGMENTS

We thank Steve Short, Paula Sherman, Thomas Spector, Dave Porter, and Jim Fyfe for stimulating and helpful discussions and Thomas Krenitsky for his continued support.

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