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Incomplete Removal of the RNA Primer for Minus-Strand DNA Synthesis by Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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A synthetic RNA oligonucleotide (15-mer) corresponding to the 3′ end of the lysine tRNA primer was hybridized to single-stranded DNA containing the human immunodeficiency virus type 1 (HIV-1) primer-binding site and extended with a DNA polymerase. The resulting structures were used to study primer removal by the RNase H activity of HIV-1 reverse transcriptase. The initial cleavage event removes the RNA primer as a 14-mer and leaves a single ribonucleotide A residue bound to the 5′ end of the DNA strand. This result explains the observation by several groups that HIV-1 circle junctions contain 4 bp that are not present in the integrated provirus instead of the predicted 3 bp. Subsequent cleavage events occur at other sites internal to the RNA molecule, and the ribonucleotide A residue on the end of the DNA strand is ultimately removed. Therefore, the biologically relevant cleavage that produces the 14-mer reflects the kinetics of the reaction as well as a specificity for nucleic acid sequence. When the RNA oligonucleotide alone was hybridized to the primer-binding site and tested as a substrate for HIV-1 RNase H, the cleavage pattern near the 3′ end of the RNA was altered.

The replication cycle of a retrovirus begins with the process of reverse transcription, which occurs in the cytoplasm of the infected cell. During this process, the single-stranded plus-sense RNA genome is copied into linear double-stranded DNA by the combined DNA polymerase and RNase H activities of the virion-associated reverse transcriptase (24). The resulting molecule is bounded at each end by a long terminal repeat (LTR) with the structure U3-R-U5, where U3 and U5 are unique sequences that derive from the 3′ and 5′ ends of the RNA genome, respectively, and R is a direct repeat present at both ends (8, 24, 25). In the next step of infection, the linear DNA intermediate moves to the nucleus where it becomes integrated into the host cell chromosomal DNA. In the systems examined so far, it has been shown that 2 bp are lost from each end of the linear intermediate during integration and that the first and last two bases of the integrated provirus are highly conserved, always being 5′-TG at the left end and CA-3′ at the right end (1, 4, 11, 24, 25). Both the nature and the number of the additional bases present at the ends of the DNA intermediate before integration are determined by the two priming events which occur during reverse transcription.

The left end of the left LTR is determined by the site at which the synthesis of plus-strand DNA begins. It has been shown in vitro in several systems, including human immunodeficiency virus type 1 (HIV-1), that plus strands initiate 2 bases before the conserved TG dinucleotide observed at the left end of the provirus (9, 12, 14, 15, 22). Analysis of circle junctions formed in vivo by blunt-end ligation of linear DNA intermediates (10, 19, 20, 21, 26) has shown that plus strands initiate at this same site in vivo. The primer for the initiation of plus strands is derived from a region of the viral RNA near its 3′ end, called the polypurine tract, and is generated by RNase H cleavage at a unique site within the polypurine tract (5, 9, 15, 17, 18, 23) (Fig. 1). RNase H is also responsible for removing the primer RNA once it has been extended by the polymerase. In the systems studied thus far, the RNA is removed intact by RNase H cleavage at the RNA-DNA junction (2, 9).

The right end of the right LTR is determined by the site at which the primer for minus-strand DNA synthesis is removed. The primer for minus-strand DNA synthesis is a tRNA molecule which is paired with the viral RNA genome near its 5′ end at a locus called the primer-binding site (PBS) (Fig. 1). In most retroviruses, the CCA-3′ end of the tRNA primer is located such that synthesis of minus-strand DNA begins 2 bases before the end that is observed in the integrated provirus (24). However, for HIV-1, the CCA-3′ end of the tRNA is located only 1 base away from the end of the right LTR in the provirus (16) (Fig. 1). If minus-strand DNA synthesis were to begin at the end of this RNA primer and if the primer were to be removed at the RNA-DNA junction as has been observed for other retroviruses (13, 24), then there would be only 1 bp beyond the conserved CA dinucleotide on the right end of the linear double-stranded DNA intermediate instead of the usual 2 bp (Fig. 1, structure I). On the basis of this model, one would predict that the circle junctions formed during HIV-1 infection would contain 3 bp not present in the provirus. However, it has been found in most cases that 4 additional bp are present in the HIV-1 circle junctions (10, 21, 26). Priming of the HIV-1 minus-strand DNA has been shown to be initiated by the addition of a deoxyribonucleotide to the CCA-3′ end of the tRNA primer (26), which eliminates the possibility that the fourth base is a deoxyribonucleotide added onto a tRNA molecule that is missing the terminal ribonucleotide A (ribo A) and ends in CC-3′. As a result, it has been predicted that the origin of the fourth base is the terminal ribonucleotide of the tRNA primer, left behind when the RNase H activity of reverse transcriptase removes the rest of the primer (Fig. 1, structure II).

To test this idea, we constructed a substrate for the RNase

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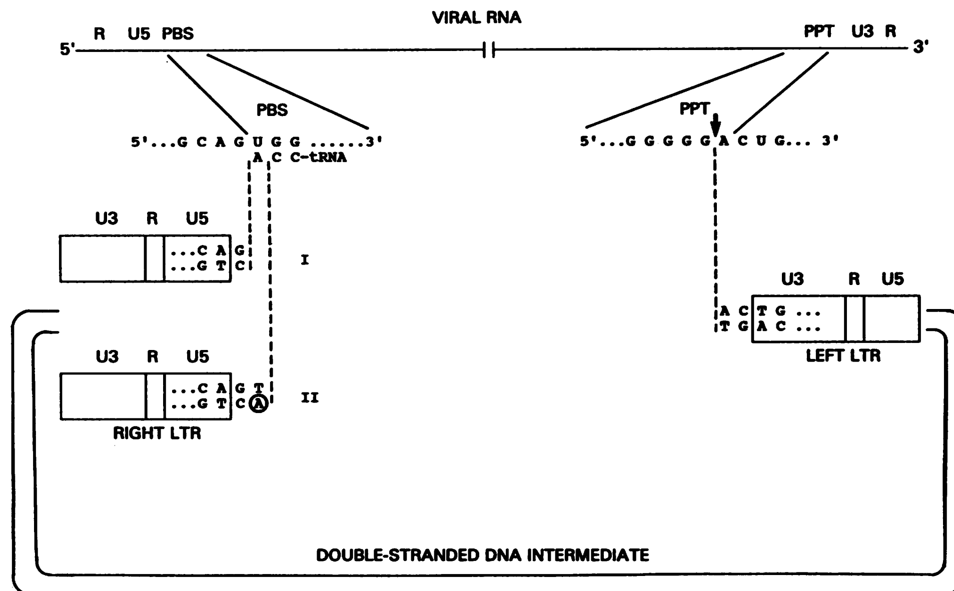

FIG. 1. Replication and integration landmarks on the retroviral genome. The site for the initiation of plus-strand synthesis within the polypurine tract (PPT) is indicated by the arrow on the right, and the resulting left LTR end is shown below. The last three nucleotides of the tRNA primer are shown paired with the PBS. The possible structures for the right LTR end are shown on the left side of the figure. Removal of the tRNA primer at the RNA-DNA junction would yield the structure labeled I, while a cleavage leaving one ribonucleotide (circled) would yield the structure labeled II. Sequences outside the boxes in the diagrams of the LTRs are the bases which must be removed during integration.

H activity of HIV-1 reverse transcriptase that mimics the in vivo substrate for primer removal. We found that reverse transcriptase does indeed make a specific cleavage such that one ribonucleotide is left on the 5′ end of the newly synthesized minus strand. Furthermore, we showed that this cleavage releases an intact primer (minus the 3′ base remaining on the DNA) and that the specificity of the cleavage is dependent upon the extension of the RNA primer into DNA. After submission of this paper, a paper by Furfine and Reardon (7) that presented very similar results appeared.

MATERIALS AND METHODS

Nucleic acids. Subclone M13mp7(T348) was constructed by inserting the *Taq*I fragment from positions 335 to 683 in HIV-1 HXB2 (16) into the *Acc*I site of M13mp7 such that the packaged single-stranded DNA contained the HIV sequences in the plus polarity. Subclone BP931 was constructed by inserting the 941-bp *Bgl*II (position 473)-to-*Pst*I (position 1414) fragment of HIV-1 HXB2 into the *Pst*I and *Bam*HI sites of M13mp19 such that the single-stranded DNA contained the HIV sequences in the minus polarity.

Oligonucleotides. The DNA oligonucleotide, designated H588+, (5′-CTAGAGATCCCTCAGACCC-3′) was synthesized with a Biosearch Model 8600 DNA synthesizer, and the 15-mer RNA primer oligonucleotide (5′-CCUGUU CGGGCGCCA-3′) was kindly supplied by Brian Reid (University of Washington). Each oligonucleotide was purified by electrophoresis in a 20% polyacrylamide gel containing 8 M urea. The bands were excised and eluted in 10 mM Tris (pH 7.5)–1 mM EDTA, and the eluate was adsorbed to a Waters Sep-Pak C-18 column. After the bands were eluted with 60% methanol–4 mM triethyl ammonium carbonate, they were stored frozen in distilled water. End-labeling reactions were carried out with 100 ng of DNA oligonucleotide H588+ or 300 ng of the RNA oligonucleotide in a 10-μl reaction mixture containing 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 50 μCi of [γ-^{32}P]ATP (New England Nuclear Corp.) and 15 U of T4 polynucleotide kinase (U.S. Biochemicals Corp.), and the mixtures were incubated at 0°C for 2 h. The oligonucleotides were precipitated twice in the presence of 2.5 M ammonium acetate with 5 volumes of ethanol and stored frozen in distilled water. The resulting specific activities were 1×10^7 cpm/μg for oligonucleotide H588+ and 1×10^6 cpm/μg for the RNA oligonucleotide.

Preparation of hybrids. For the experiments in which the site of primer removal was mapped, a 50-μl reaction mixture contained 68 ng of the M13mp7(T348) single-stranded DNA per μl, 9 ng of the unlabeled-primer RNA oligonucleotide per μl, a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 5 mM DTT. The sample was heated to 65°C for 3 min and cooled to 20°C, and 2.3 U of T4 DNA polymerase (U.S. Biochemicals Corp.) was added. After the mixture was incubated at 37°C for 30 min, the reactions were terminated with excess EDTA, and the products were precipitated in the presence of 2.5 M ammonium acetate with 2 volumes of ethanol.

For the experiments in which the fate of the RNA primer was determined, the T348 fragment was excised from the single-stranded vector with *Eco*RI as previously described (5); the resulting mixture of single-stranded vector and fragment DNA is referred to as "cut M13mp7(T348) DNA." A 10-μl reaction mixture containing 60 ng of cut M13mp7(T348) DNA per μl, 0.7 ng of the labeled RNA oligonucleotide per μl, 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 6 mM $MgCl_2$, and 1 mM DTT was heated to 65°C for 3 min to anneal the RNA primer to the DNA, and the primer was extended with 100 U of Superscript RNase H$^-$ Reverse Transcriptase (Bethesda Research Laboratories) at 37°C for

30 min under the conditions recommended by the supplier. The extension products were denatured in 97% formamide, isolated from a 5% polyacrylamide gel, and reannealed to 400 ng of the cut M13mp7(T348) DNA. The hybrids were precipitated in the presence of 0.3 M sodium acetate with 2 volumes of ethanol. To generate the hybrids in which the RNA primer was not extended, the RNA oligonucleotide was annealed to the cut M13mp7(T348) DNA as described above and purified directly by electrophoresis in a 5% polyacrylamide gel.

Reverse transcriptase reactions. The nucleic acids used in each reaction consisted of either one-fifth of the hybrid sample generated with the unlabeled RNA oligonucleotide or one-half of the gel-purified hybrid sample generated with the labeled RNA oligonucleotide. Reverse transcription reactions contained, in addition to the hybrids, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.8 mM DTT, and the indicated reverse transcriptase (0.05 to 0.5 U) in a total volume of 10 μl. Recombinant heterodimeric HIV-1 reverse transcriptase was kindly provided by Lawrence Loeb (University of Washington), the avian myeloblastosis virus (AMV) reverse transcriptase was purchased from Life Sciences Inc., and the Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase was purified from virions as previously described (5). For the mapping experiments, reaction mixtures were incubated at 37°C for the specified times, the reactions were stopped with excess EDTA, and the samples were immediately precipitated, while for the experiments in which the fate of the RNA primer was determined, 3.3-μl aliquots were removed and added to 4 μl of 97% formamide–5 mM EDTA at the indicated times.

Primer extension assay. A portion of each of the reverse transcriptase reaction mixtures containing unlabeled RNA was treated with 0.3 M NaOH for 20 min at 65°C and neutralized with 1 M acetic acid (final volume, 20 μl). A second portion of each reaction mixture was diluted to a volume of 20 μl. All samples were precipitated in the presence of 0.3 M sodium acetate with 2 volumes of ethanol. Precipitates were resuspended in distilled water, and one-half of each sample was combined with 2.5 ng of labeled DNA oligonucleotide H588+ in a final volume of 5 μl and heated to 100°C for 3 min. The reaction mixtures were brought to a volume of 7 μl such that the final conditions were 0.2 mM dNTP (for all four dNTPs), 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, and 100 U of Superscript RNase H^- Reverse Transcriptase (Bethesda Research Laboratories). After the mixtures were incubated at 37°C for 20 min, the reactions were stopped with an equal volume of a mixture containing 97% formamide, 5 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol.

Sequencing reactions. Sequence ladders were prepared with reagents from a Sequenase kit supplied by U.S. Biochemicals Corp. A 10-μl annealing mixture containing 2 μl of 5× reaction mixture, 2 μg of single-stranded BP931 DNA, and 7.5 ng of labeled DNA oligonucleotide H588+ was heated to 100°C for 2 min and cooled slowly to room temperature over 45 min. A 15-μl Sequenase reaction mixture which included 6.7 mM DTT, 33 nM dNTP (for all four dNTPs), the annealing mixture, and 7.5 U of Sequenase was prepared. After 5 min at 20°C, 3.5-μl aliquots were transferred to four tubes containing 2.5 μl of each dideoxy termination mixture. The reaction mixtures were incubated at 37°C for 5 min, and the reactions were stopped with an equal volume of Sequenase Stop Solution.

Size markers. For the experiments in which the fate of the RNA primer was determined, the sizes of the resulting fragments were compared to a size ladder generated by partial digestion of the labeled RNA oligonucleotide with nuclease P1 (P-L Biochemicals). A 10-μl reaction mixture containing 0.5 ng of the RNA oligonucleotide per μl, 50 mM sodium acetate (pH 5.5), and 25 μg of nuclease P1 per ml was incubated at 37°C for 5 min. The reaction was terminated by dilution with formamide to a final concentration of 95%.

Gel analysis. Primer extension products were analyzed on 8% polyacrylamide gels containing 8 M urea, while the products of the primer removal reactions used to monitor the fate of the labeled RNA oligonucleotide were analyzed on 20% polyacrylamide gels containing 8 M urea. In all cases, samples were heated to 90°C for 3 min in 50% formamide before being loaded on the gel. The 8% gels were dried onto Whatman 3MM filter paper before exposure to X-ray film. All autoradiograms were obtained by exposure at −70°C with an enhancing screen.

RESULTS

Primer removal leaves a ribo A on the DNA. We set up an in vitro system designed to mimic tRNA primer removal during HIV-1 reverse transcription. An RNA oligonucleotide corresponding to the last 15 bases of the lysine tRNA molecule known to prime minus-strand synthesis (16) (the PBS is actually complementary to last the 18 bp of the tRNA molecule) was hybridized to a single-stranded plus-sense DNA containing the HIV-1 PBS and extended with T4 DNA polymerase. After ethanol precipitation, the products were incubated with HIV-1 reverse transcriptase for 30 min, and a portion of each reaction mixture was treated with alkali to remove any residual RNA. A control reaction mixture was incubated without added reverse transcriptase. The 5′ ends of the products were mapped by an oligonucleotide extension assay with RNase H^- reverse transcriptase as the DNA polymerase. The products were analyzed on a denaturing polyacrylamide gel next to dideoxy-sequencing ladders generated from the same labeled oligonucleotide annealed to a minus-strand DNA template containing the HIV-1 PBS. By comparing the mobility of the primer extension products (Fig. 2A, lane 1, band a) with the sequence ladders, it can be seen that there was indeed an extra base on the end of the minus-strand DNA; the resulting sequence reads 5′-rAdCdTdG. . . . In the sample that had been treated with alkali, the product was one nucleotide shorter, confirming that the extra base was, as expected, a ribonucleotide (Fig. 2A, lanes 2 and 4; also, see Fig. 4, lanes 1 and 5, in which the recovery of the alkali-treated sample is better). In the control experiment, the end of the 15-base RNA oligonucleotide mapped to the predicted site (Fig. 2A, lane 3). It is noteworthy that no strands containing intermediate-length RNAs were detectable, suggesting that the cleavage reaction leaving the ribo A on the end of the DNA occurs more rapidly than any cleavages internal to the RNA (see below).

In the above experiment, primer removal was not complete since there was still a significant amount of substrate containing the full-length 15-base RNA primer attached to the DNA (Fig. 2A, lane 1). In order to test for complete removal of the primers, we monitored the course of the reaction for up to 2 h (Fig. 2B). It can be seen that for reaction times longer than 1 h, the primers were completely removed, as evidenced by the disappearance of the extension product corresponding to the full-length RNA primer (Fig. 2B, band b). Interestingly, at 1 h or longer, some of the products lacked the terminal ribo A residue, indicating that

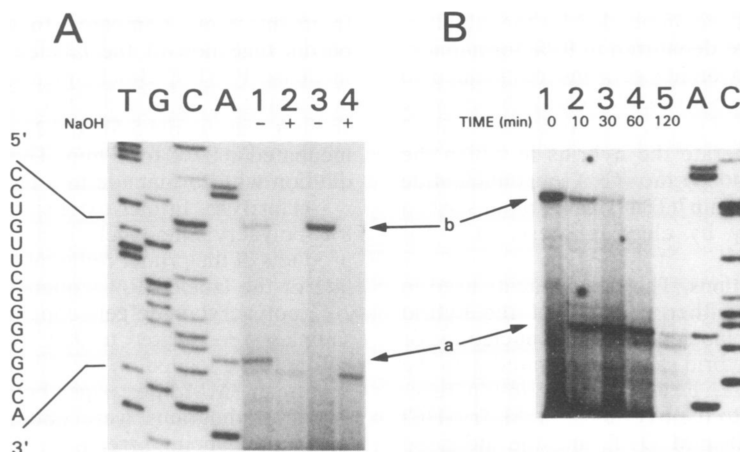

FIG. 2. Mapping the site at which the RNA primer for minus DNA synthesis is removed. (A) The RNA oligonucleotide was annealed to the single-stranded M13mp7(T348) plus-strand DNA template and extended with T4 DNA polymerase. The resulting substrate was treated with HIV-1 reverse transcriptase for 30 min at 37°C, and the 5′ ends of the products were mapped by a primer extension assay with 5′-end-labeled oligonucleotide H588+. The primer extension products were separated on an 8% polyacrylamide–8 M urea gel next to dideoxy-sequencing ladders generated by using the same labeled oligonucleotide (lane 1). The products from a parallel reaction were treated with 0.3 M NaOH prior to the primer extension assay to completely remove any ribonucleotides still attached to the DNA strand (lane 2). Control reactions were carried out in which the reverse transcriptase was omitted, and the products were analyzed directly by the primer assay (lane 3) or treated with 0.3 M NaOH before the assay (lane 4). (B) Same as for panel A except that the reverse transcription reactions were allowed to proceed for the indicated times. The arrow labeled a shows the positions in the gels of the primer extension products resulting from minus strands that retain one ribonucleotide on their 5′ ends. The arrow labeled b shows the positions of the primer extension products that extend to the 5′ end of the RNA oligonucleotide. The sequence of the RNA oligonucleotide is shown on the left side of panel A.

RNase H can slowly cleave at the RNA-DNA junction (Fig. 2B, lanes 4 and 5).

The RNA primer is removed intact. To determine the fate of the RNA primer during the reaction, we employed a system similar to the one described above. However, in this case, the 5′ end of the RNA oligonucleotide was labeled, and the T348 fragment containing the HIV-1 PBS was excised from the M13mp7 vector before the hybrids with the labeled RNA primer were formed. The resulting hybrids were treated with RNase H^- reverse transcriptase to extend the RNA primer to the end of the DNA template and gel purified to remove the excess RNA oligonucleotide (see Materials and Methods). The products were treated with HIV-1 reverse transcriptase for various lengths of time and analyzed on a denaturing polyacrylamide gel next to a size ladder generated by partial digestion of the labeled RNA oligonucleotide with nuclease P1. The major product at 30 and 75 s was a 14-mer (Fig. 3A, lanes 2 and 3), the appearance of which coincided with the disappearance of the slowly migrating band that corresponded to the untreated, extended oligonucleotide. Therefore, the initial cleavage event in the RNA removes the primer RNA intact except for the terminal A residue, which, as shown above, remains attached to the DNA. By 10 min, the 14-mer was no longer observed and smaller fragments appeared, indicating that additional cleavages had occurred internal to the RNA fragment.

To test whether the cleavage pattern observed above requires the presence of the deoxyribonucleotides attached to the 3′ end of the RNA, similar reactions in which the RNA oligonucleotide alone was annealed to the template DNA were carried out. In these experiments, the major product at the earliest time points was a 13-mer, and the 14-mer was never observed. A pattern of smaller fragments which matched that observed for the extended RNA oligonucleotide was also present at each time point.

The rate of primer removal with the labeled RNA oligonucleotide is significantly greater than the rate observed in the experiments described above, in which primer removal was measured by the primer extension assay. It is likely that this difference is due to the fact that the total DNA concentration in the experiments using the labeled RNA was approximately 10-fold lower than that in the earlier experiments.

HIV-1 primer removal by other reverse transcriptases. To determine whether the specificity observed for the HIV-1 reverse transcriptase with the HIV-1 sequence resides in the enzyme or in the substrate, the same construct was tested as a substrate for primer removal by both the AMV and Mo-MuLV reverse transcriptases. The results shown in Fig. 4 indicate that both enzymes cleave at the same site as HIV reverse transcriptase, leaving the 3′-terminal ribonucleotide of the RNA oligonucleotide attached to the minus-strand DNA.

DISCUSSION

The RNase H activity of reverse transcriptase plays several critical roles in the retroviral life cycle. (i) Degradation of the RNA hybridized to DNA removes the RNA genome once it has served its purpose as a template for plus-strand DNA synthesis (24), (ii) specific cleavage of the RNA genome generates the primer for plus-strand synthesis (5, 9, 14, 15, 18, 23), and (iii) specific cleavages release the RNA primers from the nascent plus- and minus-strand DNAs (2, 9, 13). In the retroviral systems studied thus far, removal of both the minus- and plus-strand primers occurs at the junction between the RNA and the DNA and generates LTR ends with 2 bp that are symmetrically lost during integration (24). The analysis of HIV-1 circle junctions (10, 21, 26) as well as in vitro studies of plus-strand priming (9, 15) has shown that the plus-strand primer for HIV-1 is similarly removed by cutting at the RNA-DNA junction to

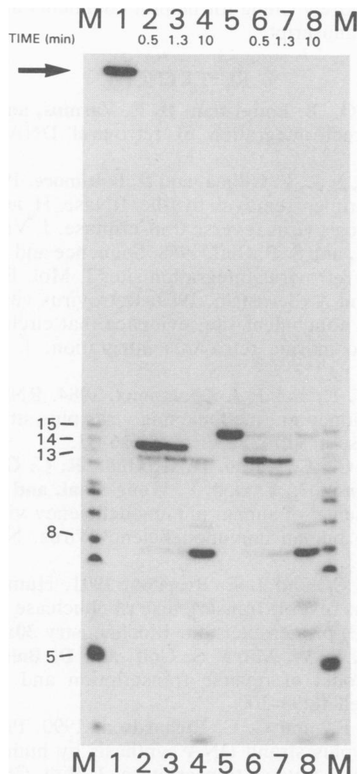

FIG. 3. The fate of the RNA primer. The labeled RNA oligonucleotide primer was annealed to the single-stranded cut M13mp7 (T348) plus-strand DNA template and extended with RNase H$^-$ reverse transcriptase. The products were gel purified and treated with HIV-1 reverse transcriptase for 30 s (lane 2), 75 s (lane 3), and 10 min (lane 4). A control reaction in which reverse transcriptase was omitted (lane 1) was carried out. The arrow at the top left marks the position of the extended RNA primer seen in lane 1. A series of parallel reactions in which the annealed RNA oligonucleotide primer had not been extended were carried out. The reactions with reverse transcriptase were allowed to proceed for 30 s (lane 6), 75 s (lane 7), and 10 minutes (lane 8). The reverse transcriptase was omitted from the control reaction (lane 5). The ladder of size markers was generated by partial digestion with nuclease P1 (lanes M). The lengths (in nucleotides) of several of the marker fragments are indicated on the left.

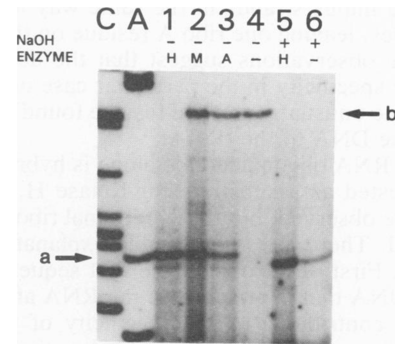

FIG. 4. Mapping the site at which the RNA primer for minus-strand DNA synthesis is removed by different reverse transcriptases. The reactions and primer extension analyses were carried out as described for Fig. 2, with HIV reverse transcriptase (H) (lanes 1 and 5), Mo-MuLV reverse transcriptase (M) (lane 2), and AMV reverse transcriptase (A) (lane 3). Control reactions were carried out without any added reverse transcriptase (lanes 4 and 6). The samples shown in lanes 5 and 6 were treated with NaOH prior to the primer extension analysis. The designations for the arrows labeled a and b are the same as for Fig. 2.

generate a left LTR end with 2 bp beyond the conserved TG dinucleotide. For the right LTR end, however, removal of the minus-strand primer for HIV-1 at the RNA-DNA junction would leave only 1 bp beyond the conserved CA dinucleotide; however, this is contrary to the circle junction data. We show here that the RNase H activity of the HIV-1 reverse transcriptase removes the minus-strand primer by a specific cleavage that leaves the 3′-terminal ribonucleotide attached to the newly synthesized DNA strand. Thus, the right LTR end would have the usual 2 bp beyond the CA dinucleotide, except in this case the 5′-terminal base is a ribonucleotide instead of a deoxyribonucleotide.

An in vitro analysis of the kinetics of the cleavage reaction indicates that the minus-strand primer is removed intact by a single cleavage between the C and the 3′-terminal A residues of the tRNA molecule. Other cleavage events internal to the RNA become detectable as the reaction progresses, presumably because the RNA primer remains annealed to the DNA template after the initial cleavage and continues to serve as a substrate for the cleavage activity of RNase H. Whether these secondary cleavages occur during HIV-1 infection or whether the initial cleavage is sufficient to completely dissociate the tRNA from the DNA is unclear. In the case of the avian retroviruses, it has been shown that the initial cleavage event at the RNA-DNA junction uncouples the tRNA from the minus-strand DNA and releases the tRNA intact without any secondary cleavage events (13).

A kinetic analysis similarly reveals that the RNA-DNA junction itself is not absolutely resistant to the action of RNase H, since after prolonged incubation, the DNA strands that accumulate lack a 5′-terminal ribonucleotide. Since virtually all of the substrate is converted to strands containing the 5′-ribo A before significant amounts of the RNA-free DNA strands are observed (Fig. 2B), it appears that the single ribo A residue on the end of the minus-strand DNA provides a suitable, albeit inefficient, substrate for RNase H. Apparently, removal of the terminal ribo A residue can also occur during infection but is likely to be a rare event since only a few circle junctions with the corresponding structure have been observed (10, 21). Presumably, the events that follow reverse transcription, including integration and formation of circle junctions by blunt-end ligation, occur rapidly enough in the infected cell that the terminal ribo A residue is retained. Although unlikely, it is also possible that circle junctions do not accurately reflect the distribution of structures for the unintegrated DNA intermediates and that the right LTR terminus lacking ribonucleotides is the true substrate for integration.

What is the molecular basis for the relative resistance of the terminal ribo A residue on the minus-strand DNA to the HIV-1 RNase H activity? It is unlikely that the HIV-1 RNase H is intrinsically unable to cleave at an RNA-DNA junction, since plus-strand primers are removed completely and efficiently (9). In addition, the avian enzyme has previously been shown to remove its own tRNA primer by cutting precisely at the junction between the RNA and the DNA (13), and similar results have been described for the removal of the plus-strand primers by both the AMV and Mo-MuLV enzymes (2, 17). Nevertheless, both of these enzymes preferentially cleave the substrate designed to mimic the HIV-1

RNA-primed minus strands in the same way as the HIV-1 RNase H does, leaving one ribo A residue on the end of the DNA. These observations suggest that the determinant of the cleavage specificity in the particular case of HIV-1 may reside in some unusual structural feature found at the site of linkage of the DNA to the tRNA.

When the RNA oligonucleotide alone is hybridized to the DNA and tested as a substrate for RNase H, the internal cleavages are observed, but the 3'-terminal ribo A residue is not removed. There are two possible explanations for this observation. First, it is conceivable that sequence information in the DNA that is attached to the RNA after it is used as a primer contributes to the specificity of the cleavage reaction. This possibility seems unlikely, given the specificity of RNase H for DNA-RNA hybrids, but cannot, at present, be ruled out. Second, the terminal ribo A residue in the unextended RNA hybrids may be only loosely base paired with the DNA and, for this reason, may not be recognized as a hybrid by RNase H. This situation differs from that of the extended hybrids in which the tRNA terminus should be tightly paired to the DNA and, thus, be a suitable substrate for RNase H. Interestingly, the production of the 13-mer product is enhanced in the reaction with the RNA alone compared with the production in the reaction with the extended primer. The basis for this observation is unclear.

Our finding that RNase H leaves an extra ribo A residue on the 5' end of minus strands provides an explanation for the observation that most HIV-1 circle junctions contain 4 rather than the predicted 3 bp between the conserved CA and TG dinucleotides (10, 21, 26). Furthermore, since the initiation site for minus strands determines the end of the right LTR, which, in turn, is one of the substrates for integration, our findings suggest that the 2 bp beyond the conserved TG and CA dinucleotides may form the preferred substrates for integration. There is yet another example in HIV-1 replication in which primer removal by RNase H generates such a substrate. In vitro, initiation of plus strands occasionally occurs at several sites downstream of the normal site (9, 15). When this happens, primer removal by RNase H still leaves 2 bases before the TG dinucleotide, even though one or both of these bases may be ribonucleotides (9). Thus, as for most of the other known retroviruses, it appears that the presence of 2 bp beyond what will be the HIV-1 proviral ends may be important for the replication cycle, possibly to provide a symmetrical substrate for integration. However, for Mo-MuLV, it has been shown that mutants with quantitative alterations of the right LTR end can still integrate in vivo (3). Furthermore, for HIV-2 and simian immunodeficiency virus (MM251), the tRNA primer is located such that 3 bp beyond the conserved CA dinucleotide should remain on the right end of the right LTR (6, 27). For these viruses, either the plus-strand start site is located at an unusual location that leaves the 3 bases on the left LTR end, or the substrate for integration is asymmetrical, with 2 bp beyond the TG dinucleotide on the left end and 3 bp beyond the CA dinucleotide on the right end of the DNA intermediate. Further studies are required to discriminate between these alternatives, but these considerations do suggest that symmetry in the integration reaction, while preferred, may not be absolutely required.

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