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Sequence Requirements for Removal of tRNA by an Isolated Human Immunodeficiency Virus Type 1 RNase H Domain

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Retroviral reverse transcriptase-associated RNase H enzymes are responsible for degradation of viral RNA, including removal of the tRNA primer after plus-strand strong-stop synthesis and cleavage of the polypurine tract primer. These activities are required for the complex viral replication and result in generation of the long terminal repeats. The human immunodeficiency virus type 1 (HIV-1) RNase H domain has been expressed independently of the polymerase domain and possesses Mn^{2+}-dependent activity with a hexahistidine tag. The isolated domain maintains the ability to specifically remove a tRNA primer mimic. In this study, the substrate determinants for recognition of the cognate $tRNA_3^{Lys}$ are defined. Model substrates were constructed which mimic the RNA-DNA hybrid obtained from plus-strand strong-stop synthesis. Deletion substrates containing only 12, 9, or 6 positions of the tRNA primer were capable of being cleaved by the isolated RNase H domain. Mismatch and bromodeoxyuridine mutagenesis analysis indicated that positions 2, 3, 4, and 6, when mutated, affected the specificity of RNase H activity. Substitution substrates indicated that positions 4 and 6 within the RNA primer were important for recognition and cleavage by the HIV-1 isolated RNase H domain. Moloney murine leukemia virus-HIV-1 hybrid substrates were constructed which demonstrated that changes to HIV-1 sequences at positions 4 and 6 were sufficient but not optimal for regaining cleavage by the isolated HIV-1 RNase H domain. Optimal site-specific cleavage between the terminal ribonucleotide A and ribonucleotide C requires additional sequences beyond the first six positions but less than nine.

Retroviruses contain an RNA genome which is reverse transcribed into a DNA copy by the viral reverse transcriptase (RT). RT is a multifunctional enzyme containing RNA- and DNA-dependent DNA polymerase activities and RNase H activity. Replication of the virus requires RNase H activity (20, 24, 33, 34) to remove the RNA within RNA-DNA hybrid intermediates formed during the polymerization process. The double-stranded DNA product is subsequently randomly integrated into the host's genome (2).

Although the viral RNase H is required for the removal of the RNA throughout the replicating genome, two specific cleavages are critical. The first involves the generation of the plus-strand primer (21). The second is the removal of the tRNA primer used in minus-strand DNA synthesis (4, 14). During viral replication, the first 18 nucleotides of the tRNA are reverse transcribed, regenerating the primer binding site (PBS) during synthesis of plus-strand strong-stop DNA. Removal of the tRNA from the RNA-DNA hybrid by RNase H exposes the repeat sequence required for the second strand transfer reaction. Imprecise removal of the tRNA primer could result in long terminal repeat termini, required for the subsequent integration of the viral DNA, being incorrect. The removal of the tRNA primer has been characterized for human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus (M-MuLV) RTs. For HIV-1, the RT-RNase H cleaves the $tRNA_3^{Lys}$ between the terminal ribonucleotide A

and ribonucleotide C (18, 32), leaving a terminal ribonucleotide which is stable in vivo (8, 9, 12, 16, 30). Interestingly, M-MuLV RT initially cleaves the $tRNA^{Pro}$ at the identical position, between the terminal ribonucleotide A and ribonucleotide C of the tRNA (27, 28). In contrast, this terminal ribonucleotide is subsequently removed and is not found associated in the circle junctions isolated from infected cells (28).

HIV-1 RT consists of a heterodimer of p66 and p51 subunits. The RNase H domain is encoded at the C terminus of p66, and the polymerase domain is at the N terminus. Evidence of the interactions between these domains is established. RNase H activity has been characterized as being either polymerase dependent or polymerase independent (1). In the polymerase-dependent mode, footprinting analysis has determined that the RNase H active site lags approximately 18 to 20 nucleotides behind the actively synthesized polymerase domain (38–40). Mutations within the primer grip region of the HIV-1 polymerase domain have been shown to have decreased RNase H activity and specificity, indicating the interrelations of these two domains (6, 15, 17). Conversely, alterations in RNase H have been shown to destabilize the interactions between RT and the primer template (7, 19).

Isolated retroviral RNase H domains separated from the polymerase domains have been constructed. This allows for the direct analysis of RNase H cleavages independent of the polymerase activity. The isolated RNase H domain of M-MuLV was not capable of specific RNase H cleavages in the absence of the polymerase domain (26, 41). A series of HIV-1 isolated RNase H domains which are active in manganese have been constructed with or without a histidine tag (29, 31). The isolated HIV-1 RNase H domain cleaves model substrates for $tRNA_3^{Lys}$ primer removal at the same specific position as the RT-associated RNase H domain (31).

This study focuses on characterizing polymerase-independent RNase H activity catalyzed by an isolated HIV-1 RNase

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H domain. Current research shows that an isolated HIV-1 RNase H domain is capable of distinguishing a tRNA^{Pro} mimic from a tRNA$_3^{Lys}$ mimic for primer removal. Model substrates have been constructed possessing an RNA primer consisting of the first 18 nucleotides of tRNA$_3^{Lys}$. This substrate models the viral replication intermediate after plus-strand strong-stop DNA synthesis. Deletion substrates have been constructed to determine the minimal sequences necessary for removal by the HIV-1 RNase H. Mismatch, substitution, and hybrid substrates were constructed to characterize the specific sequences important for recognition. The data indicates that sequences outside the scissile bond, through the first 8 nucleotides of the tRNA, are important for optimal and specific removal of the tRNA.

MATERIALS AND METHODS

Enzymes and nucleotides. T4 polynucleotide kinase was purchased from New England Biolabs; recombinant RNasin was purchased from Promega. Exonuclease(−) Klenow polymerase was purchased from United States Biochemical. HIV-1 RT was obtained from Jeffrey Culp and Christine Debouck, Department of Protein Biochemistry, SmithKline Beecham Pharmaceuticals. HIV-1 isolated RNase H (NY427) was purified from *Escherichia coli* containing the plasmid pET-NY427 (31). NY427 encodes an N-terminal hexahistidine tag and contains Mn^{2+}-dependent RNase H activity. [γ-^{32}P]ATP was purchased from ICN.

Oligonucleotides. The RNA-DNA oligonucleotides 17 mer (5′ **GUUCGGGC GCCA**CTGCT 3′), 14 mer (5′ **CGGGCGCCA**CTGCT 3′), and 11 mer (5′ **GCG CCA**CTGCT 3′) were synthesized by Integrated DNA Technologies (RNA sequences are indicated in boldface). The RNA-DNA oligonucleotides position 3 (5′ **GUUCGGGCGUCA**CTGCT 3′), position 6 (5′ **GUUCGUCGCCA**CTGCT 3′), position 4 and 6 (5′ **GUUCGGUCUCCA**CTGCT 3′), MuLV-HIV hybrid 4&6 (5′ **GACGAGGCGCCA**TTACT 3′), MuLV-HIV hybrid 4,6,&8 (5′ **GACG GGGCGCCA**TTACT 3′), and MuLV-HIV hybrid 4,6,8,&9 (5′ **GACCGGGCGC CAT**TACT 3′) were purchased from the DNA Synthesis Laboratory, Department of Biochemistry, University of Medicine and Dentistry of New Jersey (UMDNJ). The RNA oligonucleotides MPR-1 (5′ **ATCCCGGACGAGCCCCCA** 3′) and HPR-1 (5′ **UCCCUGUUCGGGGGCGCCA** 3′) were synthesized by Integrated DNA Technologies. The DNA oligonucleotides 5331 (5′ AGCAGTGG CGCCCGAACGCGGGGCTTGTCCCT 3′), 6899 (5′ TCATTTGGCGCCCC GTC 3′), 6956 (5′ TCATTTGGCGCCCGGTC 3′), 7900 (5′ TCATTTGGCGC CTCGTC 3′), 6583 (5′ AGCAGTGGCGTCCGAAC 3′), 6582 (5′ AGCAGTG TCGCCCGTTC 3′), 6523 (5′ AGCAGTGGTGTCCGTTC 3′), 5193 (5′ GTCA GCGGGGGGTCTTTCATTTGGGGGCTCGTCCGGGAT 3′), and HTD-1 (5′ GTGTGGAAAATCTCTAGCAGTGGCGCCCCGAACAGGGA 3′) were synthesized by the DNA Synthesis Laboratory, Department of Biochemistry, UMDNJ. The following BrdU oligonucleotides were purchased from the DNA Synthesis Laboratory, Department of Biochemistry, UMDNJ (the position mutated to BrdU is indicated by an X): 6429 (5′ AGCAGXGGCGCCCGAAC 3′), 6435 (5′ AGCAGTGXCGCCCGAAC 3′), 6428 (5′ AGCAGTGGXGCCCG AAC 3′), 6427 (5′ AGCAGTGGCGXCCGAAC 3′), 6426 (5′ AGCAGTGGC GCXCGAAC 3′), and 6425 (5′ AGCAGTGGCGCCXAAC 3′). The complementary DNA strands utilized in the mismatch assays were 6388 (5′ AGCAGT AGCGCCCGAAC 3′), 6341 (5′ AGCAGTGACGCCCGAAC 3′), 6387 (5′ AGCAGTGGAGCCCGAAC 3′), 6342 (5′ AGCAGTGGCACCCGAAC 3′), 6386 (5′ AGCAGTGGCGACCGAAC 3′), and 6385 (5′ AGCAGTGGCGCAC GAAC 3′).

tRNA removal substrate preparation. Substrates were prepared as previously described (28). Briefly, 20 pmol of each substrate was 5′ end labeled with [γ-^{32}P]ATP, gel isolated, and eluted overnight (28). The labeled substrate was annealed to 20 pmol of the indicated annealing strand in a 30-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl$_2$, and 2 mM dithiothreitol (DTT). The mismatch substrates were prepared in the same manner. The 17 mer RNA-DNA hybrid was 5′ labeled and annealed to an oligonucleotide which would create a mismatch at the indicated position. The bromodeoxyuridine (BrdU)-mutagenized substrates were chemically synthesized to contain a BrdU at the indicated position. These DNA substrates were then annealed to the 17 mer RNA-DNA hybrid substrate as described above.

RNase H cleavage assays. The annealed, RNA-DNA hybrid substrates were incubated with either HIV-1 RT, M-MuLV RT, or NY427 (an isolated RNase H domain) (31). Reaction mixtures (20 μl) contained approximately 4 pmol of substrate. The HIV-1 RT and M-MuLV RT reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, and 8 mM MnCl$_2$. NY427 reaction mixtures contained *N*-morpholinoethanesulfonic acid (MES), pH 6.4, 0.2 mM DTT, and 8 mM MnCl$_2$. For each experiment, 1 pmol of enzyme was used unless otherwise indicated. The reaction mixtures were all incubated at 37°C. Aliquots (3 μl) were removed at 0, 2, 5, 15, and 30 min. The reactions were stopped by the addition of formamide stop buffer. The reaction products were separated on 20% denaturing polyacrylamide gels.
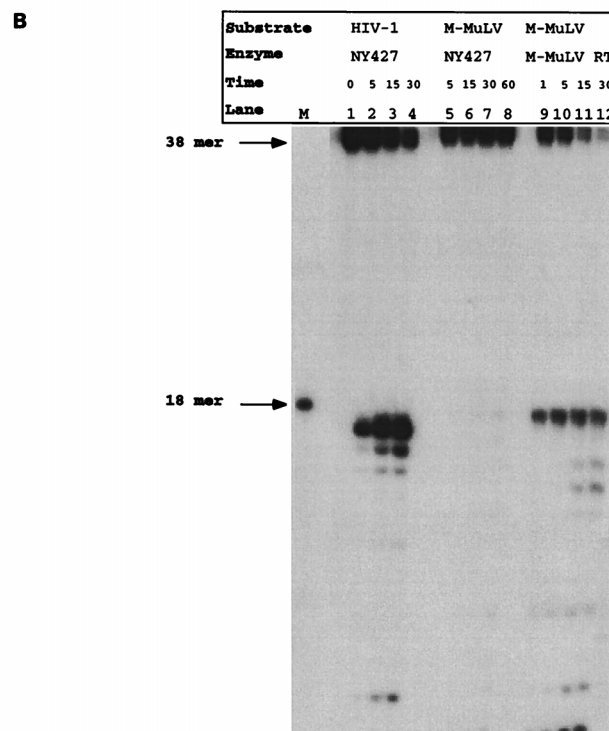

FIG. 1. Sequence-specific tRNA removal by an isolated RNase H domain. (A) Model substrates utilized to mimic intermediates of M-MuLV and HIV-1 reverse transcription. The RNA portion of each substrate is indicated in boldface. The asterisk denotes the 5′ label. The RNA oligonucleotide was 5′ labeled with [γ-^{32}P]ATP, annealed to a complementary DNA oligonucleotide, and extended with Exonuclease (−) Klenow polymerase. The resultant RNA-DNA hybrid was used in an RNase H cleavage assay. (B) RNase H cleavage assay of NY427, an HIV-1 isolated RNase H domain, with M-MuLV and HIV-1 model substrates. Lanes 1 to 5, NY427 with HIV-1 model substrate; lanes 6 to 10, NY427 incubated with M-MuLV model substrate; lanes 11 to 15, M-MuLV model substrate incubated with M-MuLV RT. Lane M contains the RNA marker, which is an 18-mer. Time points are indicated in minutes above each lane.

RESULTS

Sequence-specific tRNA removal by an isolated RNase H domain. Figure 1A illustrates the model substrates utilized to characterize the specific cleavages of the isolated HIV-1 RNase H domain, NY427. The substrates model an intermediate of reverse transcription after plus-strand strong-stop synthesis. At this point, the first 18 nucleotides of the tRNA has been reverse transcribed, yielding an RNA-DNA hybrid. The tRNA primer is then removed by the RNase H domain of HIV-1 RT. The two model substrates utilized in this assay mimic either the M-MuLV intermediate containing tRNA^{Pro} or an HIV-1 intermediate, which utilizes tRNA$_3^{Lys}$. The RNA oligonucleotides are 5′ labeled, annealed to the complementary DNA strand (38 nucleotides long), and extended with Exonuclease(−) Klenow polymerase.

The recognitions of these RNA-DNA hybrid substrates by the NY427 RNase H domain (31) of HIV-1 RT were compared (Fig. 1B). Previous analysis of NY427 indicated that this isolated RNase H domain removes the HIV-1 tRNA primer

with the same specificity as the p66-p51 full-length RT het-
erodimer (31), with the initial cleavage occurring between the
3′ C and A of the RNA moiety. With the model substrates
shown in Fig. 1A, this specific cleavage would release a 5′-
labeled 17-mer product. Figure 1B, lanes 1 to 4, shows a time
course of the isolated NY427 with the cognate HIV-1 sub-
strate. The 17 mer oligoribonucleotide is released as the initial
and predominant product. This corresponds to cleavage be-
tween the 3′ C and A. In contrast, the HIV-1 isolated RNase
H domain was incapable of cleaving the MuLV tRNA^Pro
mimic model substrate (Fig. 1B, lanes 5 to 8). Control exper-
iments indicated that the MuLV tRNA^Pro mimic substrate can
be recognized by the MuLV RT-RNase H (Fig. 1B, lanes 9 to
12), verifying that the substrate is a functional RNA-DNA
hybrid. The additional cleavage products observed represent
the subsequent degradation of the RNA primer by the respec-
tive enzymes (Fig. 1B, lanes 3 to 4 and 11 to 12) (25). These
results indicate that the isolated HIV-1 RNase H can discrim-
inate between model substrates for the cognate and heterolo-
gous tRNAs.

Deletion analysis of model tRNA primer. The ability of the
isolated RNase H domain, NY427, to distinguish the first 18
nucleotides of the tRNA$_3^{Lys}$ from those of tRNA^Pro implies a
sequence of structural recognition of the substrate. The sub-
strate requirements were therefore further defined. Previous
data has shown that specific tRNA cleavage requires that the
RNA be linked to DNA through a phosphodiester bond (32),
with 5 DNA nucleotides being sufficient for specific removal of
the tRNA primer mimic. Substrates were constructed which
varied in the length of the RNA portions. Substrates contained
either 12, 9, or 6 bases of the RNA primer plus 5 nucleotides
of DNA and were termed 17 mer, 14 mer, and 11 mer, respec-
tively (Fig. 2A). The RNA-DNA strands in these experiments
were chemically synthesized, and the RNA portions were 5′
labeled with [γ-^{32}P]ATP and annealed to the complementary
strands. This protocol greatly facilitates the synthesis of the
substrates and eliminates the need for extension with DNA
polymerases.

Figure 2B represents a time course of NY427 with the RNA
deletion substrates. The 17 mer was capable of being cleaved
by the isolated RNase H domain and produced a specific
cleavage product between the first and second ribonucleotides
(Fig. 2B, lanes 1 to 5). The 14 mer released an 8-mer-size
product with kinetics similar to those of the 17 mer, corre-
sponding with cleavage between the terminal ribonucleotides
C and A (Fig. 2B, lanes 6 to 10). The kinetics of cleavage of 11
mer was much slower than those of 14 mer or 17 mer (Fig. 2B,
lanes 11 to 15). A low level of product corresponding in length
to 5-mer nucleotides was released after 15 min. This indicates
that the six ribonucleotides are sufficient, but not optimal, for
recognition by the isolated RNase H domain. HIV-1 RT was
able to cleave these substrates at identical positions (data not
shown). The site of cleavage was confirmed by comparison with
E. coli RNase H digestion, which cleaves 1 nucleotide down-
stream from an RNA-DNA junction (3). Both *E. coli* RNase H
and NY427 yielded identical initial products corresponding to
the expected sizes according to an RNA ladder (data not
shown). The deletion substrates were also incubated for 30 min
in Mn^{2+} reaction buffer; the breakdown products are identical
to those found at the zero time point (data not shown).

Cleavage analysis of mismatch substrates. The deletion sub-
strate analysis indicated that, minimally, the first six ribonucle-
otides were important for recognition and cleavage by the
isolated HIV-1 RNase H domain, NY427; however, the first 9
nucleotides were required for optimal RNase H activity. These
deletion studies, along with the comparison of HIV-1 and M-
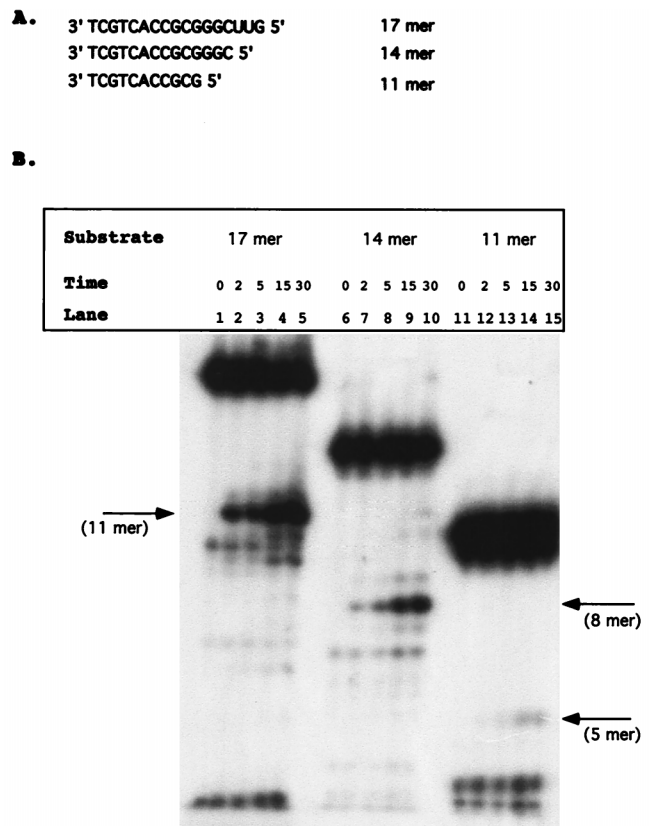

FIG. 2. Deletion analysis of model tRNA primer. (A) Substrates constructed
to analyze the effects of deleting the PBS. The substrates are termed either 17
mer, 14 mer, or 11 mer. Deletion substrates were synthesized as RNA-DNA
hybrids. The substrates were 5′ labeled with [γ-^{32}P]ATP and annealed to oligo-
nucleotide 5331 (see Materials and Methods). (B) Time course analysis per-
formed with 1 pmol of the HIV-1 isolated RNase H domain, NY427. Lanes 1 to
5, 17 mer substrate; lanes 6 to 10, 14 mer substrate; lanes 11 to 15, 11 mer
substrate. Time points are indicated in minutes above each lane. The arrows
indicate the initial cleavage product of each deletion construct.

MuLV substrates (Fig. 1), indicate there may be particular
positions which were key in recognition and cleavage by the
HIV-1 isolated RNase H domain. To further investigate this
possibility, mismatch substrates were constructed at positions
2 through 7 of the tRNA primer. The substrates were con-
structed by annealing a 5′ ^{32}P-labeled 17 mer RNA-DNA oli-
gonucleotide substrate with DNA oligonucleotides containing
an adenine (A) at position 2, 3, 4, 5, 6, or 7. Mismatches were
generated at these positions, which contain either guanidine
(G) or cytosine (C) and would not form Watson-Crick base
pairs with adenine (A) (Fig. 3A). Figure 3B shows the time
course reactions of the mismatch substrates with the HIV-1
isolated RNase H domain, NY427. Incubation of NY427 with
the wild-type substrate releases the expected 11 mer product,
indicative of cleavage occurring between the terminal ribonu-
cleotide A and ribonucleotide C (Fig. 3B, lanes 1 to 5). Mis-
matches at positions 5 (Fig. 3B, lanes 21 to 25) and 7 (Fig. 3B,
lanes 31 to 35) had no effect on RNase H activity; the release
of the product was similar to that with the wild-type substrate.
Mismatches at positions 4 (Fig. 3B, lanes 16 to 20) and 6 (Fig.
3B, lanes 26 to 30) resulted in reductions in the overall yield of
the initial cleavage product compared to the wild-type RNase
H activity. It is of interest that within the first 6 nucleotides of
the tRNA, tRNA^Pro and tRNA$_3^{Lys}$ differ at positions 4 and 6
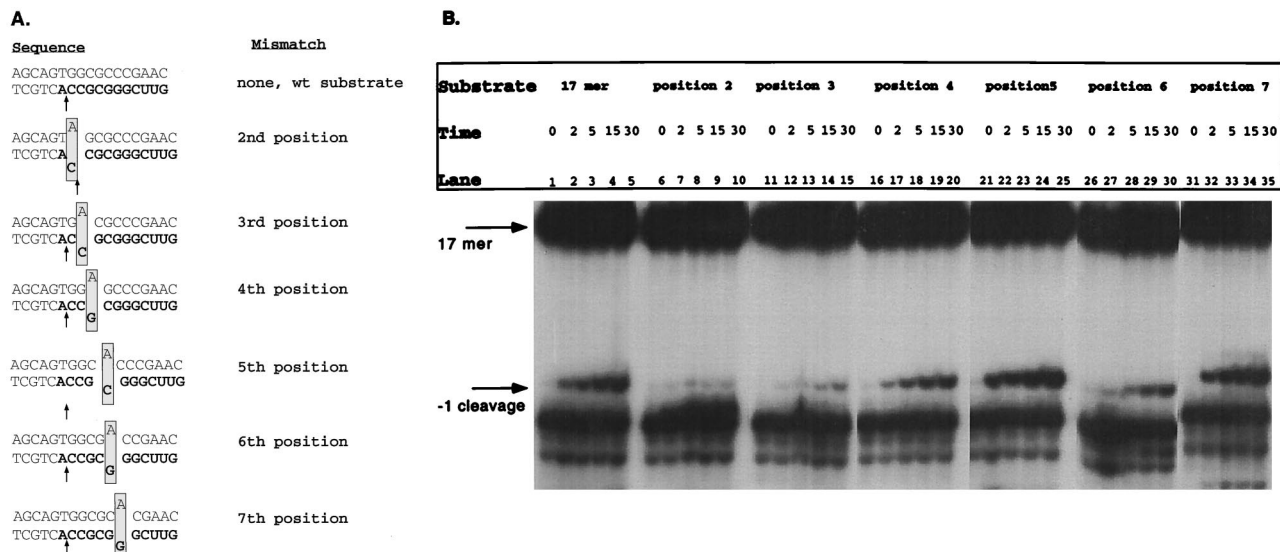(Fig. 1A). Mismatches at positions 2 (Fig. 3B, lanes 6 to 10)

FIG. 3. Cleavage analysis of mismatch substrates. (A) Wild-type (wt) and mismatch substrates utilized in this RNase H cleavage assay. The RNA-DNA hybrid substrates were 5′ labeled within the RNA portion (boldface). The hybrid was then annealed to a complementary DNA strand, which created a mismatch at a specific position. The mismatches are boxed. The annealing DNA oligonucleotides possess an adenine in position 2, 3, 4, 5, 6, or 7. The RNase H predominant cleavage observed for each substrate is indicated with an arrow. (B) RNase H cleavage assays of the mismatch substrates with the HIV-1 isolated RNase H domain, NY427 (1 pmol). Time courses were performed and are indicated in minutes above each lane. Mismatch activities (lanes 6 to 35) were compared to wild-type substrate activities (lanes 1 to 5).

and 3 (Fig. 3B, lanes 11 to 15) greatly altered RNase H activity. Cleavage normally occurs at the 3′ OH of the RNA at position 2. The mismatch at this position shifted the major cleavage site 1 nucleotide upstream, releasing an RNA product 1 nucleotide shorter than that released with the wild-type substrate. This product migrates slightly more slowly than a breakdown product of the substrate, present in the zero-time-point lanes in all of the assays (Fig. 3B, lanes 1, 6, 11, 16, 21, 26, and 31). The substrate containing a mismatch at position 3 resulted in highly diminished RNase H cleavage at the predicted cleavage site (Fig. 3B, lanes 11 to 15). These results indicated that changes at positions 2 and 3 are extremely detrimental to specific removal of the tRNA primer in an in vitro assay. These positions correspond to the CC of the CCA region, which is inherent to all tRNA primers. Along with positions 2 and 3, positions 4 and 6 produced an inhibitory effect on RNase H activity when they were in a mismatch orientation.

BrdU mutagenesis of the tRNA primer. To further analyze the sequence requirements for removal of the model tRNA primer, BrdU mutagenesis was performed on positions 1, 3, 4, 6, 7, and 8. This approach similarly generates mismatches and allows for the comparison of an adenine mismatch to a uridine mismatch. The 17 mer RNA-DNA oligonucleotide substrate was 5′ labeled with [γ-^{32}P]ATP and annealed to the complementary oligonucleotides synthesized with BrdU in either of the positions indicated in Fig. 4A. All of the BrdU substrates produced mismatches, with the exception of the one with BrdU at position 1, which can base pair with the complementary adenine. Analysis of the RNase H with BrdU at position 1 yielded maximal activity (Fig. 4B). All of the BrdU substitutions resulted in release of the 11-mer RNA product, which was quantitated as shown in Fig. 4B. The mismatch containing BrdU at position 3 had the most detrimental effect, yielding less than 6% of the cleavage of the similar substitution at position 1. Individual BrdU substitutions at positions 4 and 6 were hindered in their cleavages. Substitutions at positions 7 and 8 maintained at least 50% of the cleavage of the comple-

mentary substitution at position 1. These results confirm the mismatch results presented in Fig. 3, supporting the roles of positions 3, 4, and 6 in the recognition of the cognate tRNA-DNA substrate.

Substitution analysis of positions within the tRNA primer binding region. The mismatch data indicated that positions 2, 3, 4, and 6 were important in recognition and cleavage by the isolated RNase H domain. Further analysis aimed at creating complementary base substitutions rather than generation of mismatches. Initial analysis of substitutions of adenine, cytosine, and uracil for guanidine at position 4 indicated no changes in RNase H activity (data not shown). Therefore, substitution substrates were constructed at positions 3 and 6 and double-substitution substrates were prepared at positions 4 and 6. These substrates are illustrated in Fig. 5A. At each of the indicated positions, a uracil was substituted in the RNA portion and an adenine was substituted in the DNA portion of the substrate. This substitution in the RNA regenerates the Watson-Crick base pairing lost in the mismatch studies (Fig. 3) with base substitutions distinct from the wild-type sequences. These substrates were incubated with HIV-1 RNase H, and the initial and predominant cleavage products were compared with the wild-type substrate.

Figure 5B represents time course reactions of each substituted substrate incubated with NY427. A change at position 3 from cytosine to uracil greatly affected RNase H-specific cleavage, with a predominant cleavage product between the ribonucleotides A and G, 3 nucleotides downstream of the RNA-DNA junction (Fig. 5B, lanes 11 to 15). This indicates that position 3 is very significant for specific removal of the tRNA primer between the terminal ribonucleotide A and ribonucleotide C. The alteration at position 6 (Fig. 5B, lanes 6 to 10) had no affect on specific RNase H activity. This is similar to the single base changes at position 4, which also had no affect on RNase H activity. The double-substitution substrate at positions 4 and 6 had the most dramatic effect on specific RNase H activity (Fig. 5B, lanes 16 to 20). No specific cleavages were

A.

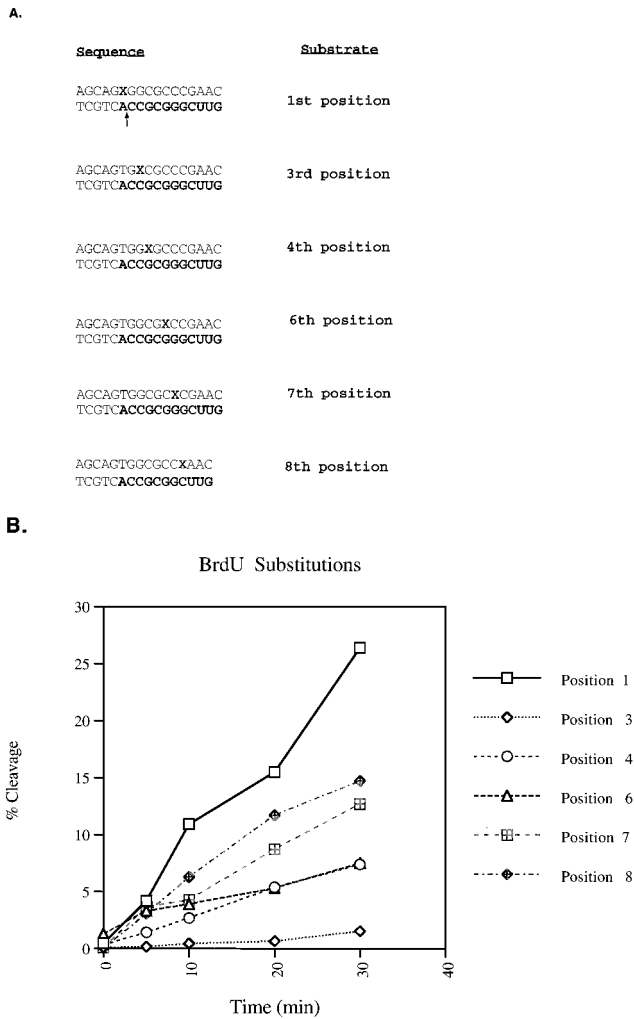| Sequence	Substrate
AGCAG**X**GGCGCCCGAAC TCGTC**ACCGCGGGCUUG**	1st position
AGCAGTG**X**CGCCCGAAC TCGTC**ACCGCGGGCUUG**	3rd position
AGCAGTGG**X**GCCCGAAC TCGTC**ACCGCGGGCUUG**	4th position
AGCAGTGGCG**X**CGAAC TCGTC**ACCGCGGGCUUG**	6th position
AGCAGTGGCGC**X**CGAAC TCGTC**ACCGCGGGCUUG**	7th position
AGCAGTGGCGCC**X**AAC TCGTC**ACCGCGGCUUG**	8th position

B.

FIG. 4. BrdU mutagenesis of the tRNA primer. (A) BrdU substrates. The substrates were synthesized with a BrdU in each position indicated by a boldface **X**. The BrdU is within the annealing strand and is annealed to a 5′-labeled RNA-DNA hybrid, 17 mer (Fig. 2). Mismatches were constructed at all positions except the second. The RNA portion of each substrate is in boldface. (B) Graphs of time course reactions of BrdU substrates assayed with the HIV-1 isolated RNase H domain, NY427. The initial cleavage products were quantified by phosphorimager analysis, and the percentage of the input substrate cleaved was determined. The legend at the right indicates the positions bearing the BrdU substitutions.

detected, only random cleavage events. This data indicates that positions 4 and 6 combined are important for recognition and cleavage by the isolated RNase H domain; however, individually they have no effect on RNase H activity.

M-MuLV–HIV-1 hybrid analysis. Results presented in Fig. 1 indicated that the isolated HIV-1 RNase H, NY427, was incapable of recognizing the M-MuLV model substrate, despite the conservation of the CCA region containing the scissile bond. Further characterization of the HIV-1 substrate had indicated a role of positions 4 and 6 in the specific recognition of the tRNA$_3^{Lys}$ mimic. It was therefore of interest to identify substitutions in the MuLV substrate which could support the recognition of this substrate by the HIV-1 RNase H.

Deletion substrate analysis (Fig. 2) indicated that the optimal recognition of the HIV substrate was within the first 12 nucleotides of the RNA. Also, the 14 mer construct possessed

kinetics similar to those of the 17 mer construct. Substitution studies as well as mismatch studies had indicated the importance of positions 4 and 6. Hybrid MuLV-HIV substrates were therefore generated in which HIV sequences were introduced into the MuLV RNA at sites between positions 3 and 9 of the RNA.

The prior substitution analysis had been performed with substrates that were 17-mers; therefore, hybrid analysis was performed with substrates of this size. Figure 6A shows the hybrid substrates constructed. The 4&6 hybrid was synthesized as a 17-mer to determine whether it would be sufficient to regain cleavage. Two additional hybrids were synthesized, 4,6,&8 and 4,6,8,&9. This allowed us to determine the optimal sequence requirements for specific recognition and removal of the RNA primer. These substrates were prepared in the same manner as the 17 mer substrate in the deletion studies.

Figure 6B shows an analysis of reactions of these hybrid substrates with the isolated RNase H domain. These reaction products were compared with those of wild-type 17 mer substrate. Each of the hybrid substrates was specifically recognized and cleaved by the isolated RNase H domain. The 4,6,&8 (lanes 11 to 15) and 4,6,8,&9 hybrids (Fig. 6B, lanes 16 to 20) possessed cleavage patterns identical to that of the wild-type 17 mer substrate (Fig. 6B, lanes 1 to 5). The 4&6 hybrid (Fig. 6B, lanes 6 to 10) was cleaved kinetically more slowly than the wild-type construct, with kinetics similar to those with 11 mer (Fig. 2). The 4&6 hybrid was confirmed to be a hybrid by digestion with *E. coli* RNase H (Fig. 6B, lanes 21 to 25).

The recognition of these substrates by the RT-associated RNase H domain was also tested. Previous data had shown that HIV-1 RT cleaves M-MuLV model substrate at the RNA-DNA junction (28), whereas it cleaves its cognate substrate between the terminal ribonucleotide A and ribonucleotide C. Reactions were performed in which these hybrid substrates

A.

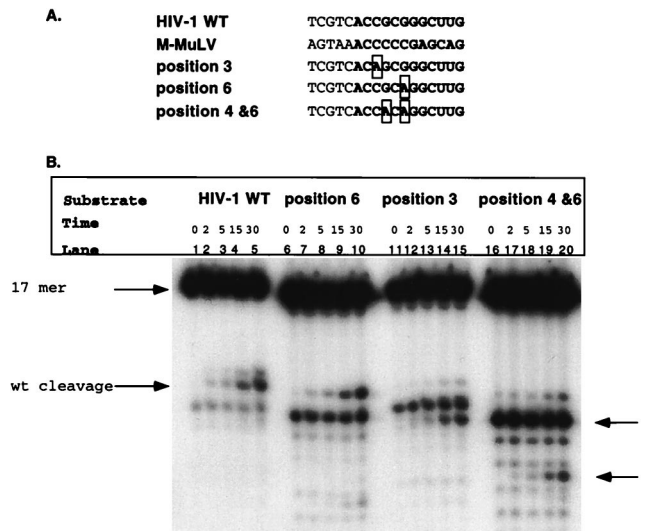|	
HIV-1 WT	TCGTC**ACCGCGGGCUUG**
M-MuLV	AGTAAA**CCCCCGAGCAG**
position 3	TCGTC**AC[A]GCGGGCUUG**
position 6	TCGTC**ACCGC[G]GGCUUG**
position 4 &6	TCGTC**ACC[A]C[G]GGCUUG**

B.

FIG. 5. Substitution analysis of positions within the tRNA primer. (A) Substitution substrates. The boxed areas indicate the positions which are changed from the wild-type (WT) sequence. The substrates were prepared as described in the legend to Fig. 4. The RNA portion of each substrate is in boldface. (B) Time course reactions of the substitution substrates. The cleavage patterns are compared to that of the wild-type substrate (lanes 1 to 5). The reactions were performed as described in Materials and Methods. Lanes 6 to 10, substitution at position 6; lanes 11 to 15, substitution at position 3; lanes 16 to 20, changes at positions 4 and 6. The time is indicated in minutes above each lane. The arrows on the right indicate altered cleavage sites, with the lower arrow indicating random cleavage events. wt, wild type.
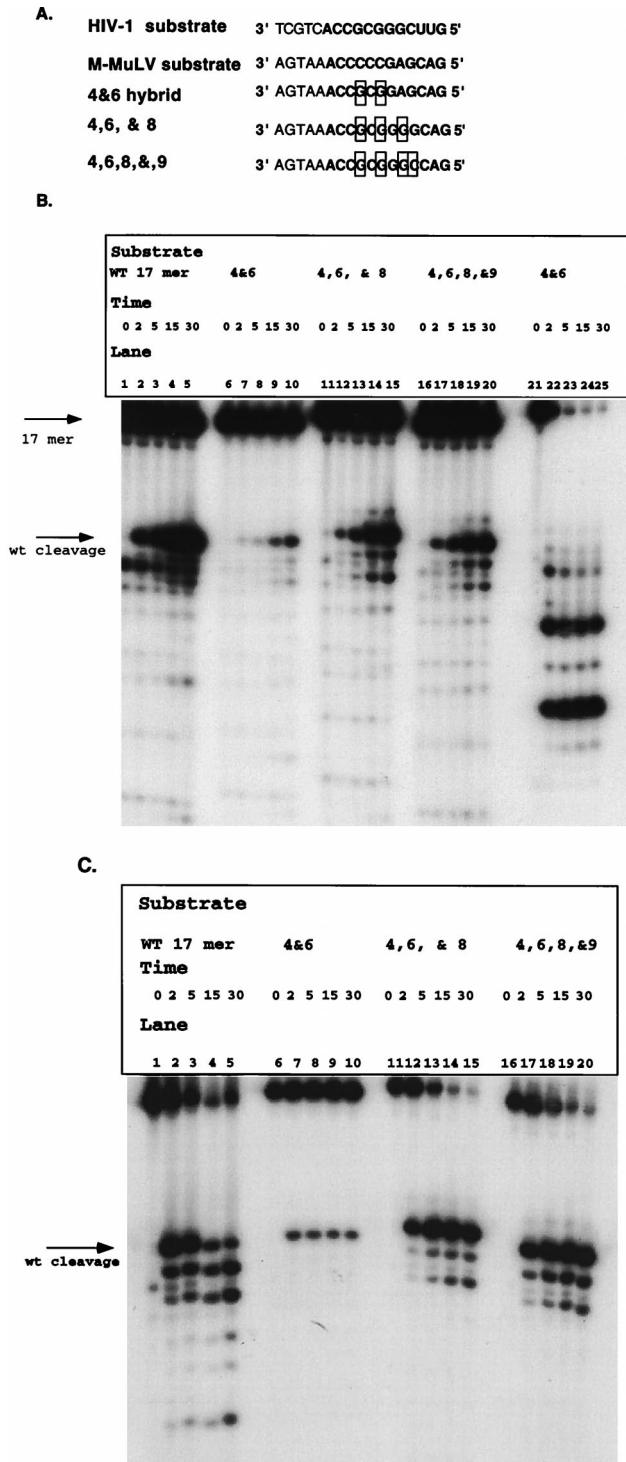
A.

HIV-1 substrate	3' TCGTCA**CCGCGGGCUUG** 5'
M-MuLV substrate	3' AGTAAA**CCCCCGAGCAG** 5'
4&6 hybrid	3' AGTAAA**CC**□**C**□**GAGCAG** 5'
4, 6, & 8	3' AGTAAA**CC**□**C**□**G**□**GCAG** 5'
4,6,8,&,9	3' AGTAAA**CC**□**C**□**G**□**□CAG** 5'

B.

C.

FIG. 6. Extensive M-MuLV–HIV-1 hybrid analysis with 17 mer construct. (A) Hybrid substrates constructed. The boxed regions are those which have been changed from the M-MuLV sequence to the HIV-1 sequence. The substrates were prepared as described in the legends to Fig. 1 to 5. The RNA portion of each substrate is in boldface. (B) Time course analysis of M-MuLV–HIV-1 hybrid substrates 4&6 (lanes 6 to 10), 4,6,&8 (lanes 11 to 15), and 4,6,8&9 (lanes 16 to 20) with the HIV-1 isolated RNase H domain. Time points (in minutes) and constructs are indicated above the lanes. Initial cleavage products, amounts, and specificities were compared to those of the wild-type (WT) 17 mer (lanes 1 to 5). Lanes 21 to 25 represent hybrid 4&6 digested with *E. coli* RNase H. (C) Analysis of RNase H cleavage of M-MuLV–HIV-1 hybrid substrate with HIV-1 RT. The hybrid substrates were incubated with 1 pmol of HIV-1 RT for 0, 2, 5, 15, and 30 min. The times and the hybrids analyzed are indicated above the lanes. wt, wild type.

were incubated with HIV-1 RT to determine if the initial cleavage product produced is 1 base larger for the hybrid substrate than for the wild-type 17 mer substrate. These reactions are shown in Fig. 6C. Hybrid 4&6 (Fig. 6C, lanes 6 to 10), hybrid 4,6,&8 (Fig. 6C, lanes 11 to 15), and hybrid 4,6,8,&9 (Fig. 6C, lanes 16 to 20) produced initial cleavage products at the same position as did the wild-type HIV-1 substrate (Fig. 6C, lanes 1 to 5). The efficiency of cleavage of 4&6 alone is lower than those of the other substrates, and no additional RNase H cleavage products were detected. Interestingly, none of the substrates produced cleavage products indicative of cleavage occurring at the RNA-DNA junction. This indicates that positions 4 and 6 in combination contribute to the recognition and cleavage of the tRNA$_3^{Lys}$ mimic.

DISCUSSION

With model substrates for plus-strand strong-stop products, the data illustrates that the HIV-1 isolated RNase H domain, NY427, is capable of selectively recognizing and cleaving substrates containing tRNA$_3^{Lys}$ sequences from those containing tRNA^{Pro} sequences. Mismatch analysis and BrdU mutagenesis provided a scanning mechanism to determine the positions important for recognition by the isolated RNase H domain. These positions were further investigated by creating substitution and hybrid substrates to directly determine the sites required to retain initial cleavage between the terminal ribonucleotide A and ribonucleotide C. Positions 2, 3, 4, and 6 were determined to be crucial in recognition. Positions 4 and 6 differ between tRNA$_3^{Lys}$ and tRNA^{Pro}.

The isolated HIV-1 RNase H appears distinct in its ability to specifically recognize its cognate tRNA for primer removal. Expression of the M-MuLV RNase H domain was reported to have little specificity (26, 41). This parallels the characteristics of the enzymes in vivo. For M-MuLV, the site of the initial cleavage is not the final cleavage product; the terminal ribonucleotide A from the tRNA is ultimately removed before completion of the viral replication (28). In contrast, the initial site of tRNA removal catalyzed by the HIV-1 RNase H is extremely stable. The terminal ribonucleotide A remains associated with the viral DNA and can be amplified within the circle junctions isolated from infected cells (8, 9, 12, 16, 30). The site of cleavage by the isolated RNase H domain is identical to that of the full-length protein. This implies an innate recognition within the RNase H domain for the sequence and/or the structure of the tRNA-DNA hybrid. This recognition cannot be ascribed to the histidine tag, since the same enzyme can differentiate between the two tRNA mimic substrates. Further studies are required to identify the domain of the protein involved in this recognition.

These studies cannot address whether it is sequence or structural recognition by the RNase H. The structure of these RNA-DNA junctions may be an important determining factor in the recognition and cleavage mechanism of RNase H enzymes. Structural analyses of related RNA-DNA hybrids indicate that the hybrids are not classic A or B structures but rather a combination of both forms, particularly around the RNA-DNA junctions (5). Studies have also shown that there is diversity in the sugar conformations at the RNA-DNA junction which may be affecting RNase H activity (23). It is quite possible that sequence can influence structure, particularly at a transition junction point. It is of interest that the identified positions which alter the cleavage recognition are at a distance from the scissile bond, up to 5 nucleotides away. This implies either that a secondary region of the RNase H rather than the active site interacts with these nucleotides or that these se-

quences greatly influence the structure of the junction. For both tRNA^{Pro} and tRNA$_3^{Lys}$, the 6 base pairs 5′ of the cleavage site are within GC base pairs. The substrates utilize an RNA primer which mimics the first 18 nucleotides of tRNA$_3^{Lys}$. The effect of any of these changes within the context of the natural tRNA$_3^{Lys}$ for tRNA removal is being explored.

The first 3 nucleotides of tRNA^{Pro} and tRNA$_3^{Lys}$ are encoded by the CCA added posttranscriptionally to all tRNAs. Alterations within this region, at position 2 or 3, either by mismatch or base substitution, altered the cleavage site or greatly reduced the efficiency of cleavage by RNase H. The CCA sequence is present in all tRNA primers, and these alterations do not explain the inability of the isolated HIV-1 RNase H domain to cleave the M-MuLV model substrate. Positions 4 and 6 defined the switch between the cognate and heterologous tRNA substrates. These were the positions which differed between M-MuLV and HIV-1 tRNAs, within the first 7 nucleotides; tRNA$_3^{Lys}$ encodes G at both positions 4 and 6, while tRNA^{Pro} contains C at both sites. In fact, insertion of AU base pairs at both these positions resulted in completely random RNase H cleavages. Single substitutions at either position 4 or 6 were tolerated. Initial studies of position 4 indicated that changes from a CG base pair to any other combination did not produce an effect. This was not systematically explored with respect to position 6.

The mismatch, BrdU mutagenesis, and substitution analyses were done with NY427 as well as HIV-1 RT. Studies with the HIV-1 RT (data not shown) have indicated that positions 3 and 6 yield alternative cleavage patterns with mismatch, substitution, and BrdU mutagenesis. Cleavage occurred predominantly between positions 3 and 4 within the tRNA primer. Substitutions at both 4 and 6 yielded cleavage at an alternative site. BrdU substitution at position 4 decreased the yield of cleavage at the predicted site. As controls, mismatches at position 5 or position 7 did not alter recognition by the full-length HIV-1 RT. These results indicate that alterations in the RNA sequences encoded in the tRNA alter the recognition of both the isolated RNase H domain and the full-length HIV-1 RT.

Hybrid studies were also performed to determine what was necessary to gain recognition and cleavage of the M-MuLV substrate by the HIV-1 isolated RNase H domain. Hybrid substrates were constructed based on the results with the deletion substrates and the substitution substrates. Other studies analyzing initiation of reverse transcription indicated that the first 6 nucleotides of the tRNA primer were required (22). Therefore, a 4&6 hybrid, as well as 4,6,&8 and 4,6,8,&9 hybrids, was constructed to determine the optimal and sufficient substrates for RNase H activity. The deletion data indicated an 11-mer was sufficient but the 14-mer was optimal. The hybrid data confirmed these results; a 4&6 hybrid was cleavable, but the 4,6,&8 and 4,6,8,&9 hybrids were cleaved more efficiently. This would indicate that for the 17 mer constructs, positions 4, 6, and 8 are important for optimal recognition and cleavage, whereas positions 4 and 6 are sufficient.

Currently, many studies point to the interactions between the polymerase and RNase H domains. Primer grip mutants which have altered RNase H activity have been isolated (6, 15, 17, 19). It would be of interest to determine if these mutations affect the specificity of the RNase H domain on small defined substrates, such as those described in this study for tRNA removal. It is possible that these mutants have diminished RNase H activity due to the inability to position the substrate within the primer-template groove. This may not alter the ability of the small targeted RNase H substrates to bind independently of the polymerase domain. Although the polymerase domain does not affect the RNase H cleavage of the cognate

tRNA, the influence on a heterologous substrate is evident. The isolated HIV-1 RNase H is not capable of recognizing the M-MuLV substrate, yet in the presence of the polymerase domain, the HIV-1 RT cleavage occurs at the RNA-DNA junction (27, 28).

Studies have been performed to assess the selection of the tRNA for initiation of reverse transcription (10, 11, 35–37). These studies have highlighted the importance of the PBS as well as a region complementary to the anticodon loop within U5. Modified viruses with altered PBSs and U5 A loops complementary to tRNA^{Pro}, tRNA^{Trp}, tRNA^{Met}, and tRNA^{His} have been found which support initiation (10, 11, 35). Slow reversion is still detected for tRNA^{Pro} and tRNA^{Trp}, indicating that the A loop and PBS are not the sole determinants for selection of specific tRNA for viral replication (11). It is interesting that of these alternative tRNAs, tRNA^{His} and tRNA^{Met} are the most stable (10, 35), and they contain 3′ termini, predicted by this study to be efficiently removed by the HIV-1 RNase H.

RNase H cleavages can occur in a polymerase-dependent or -independent mode (13, 38, 39). In the polymerase dependent mode, the RNase H cleavage lags 18 to 20 nucleotides behind the 3′ OH position in the polymerization site. In the studies described here, the cleavages occur independently of the presence of the polymerase domain. In addition, several of the truncated substrates maintain distances from the potential 3′ OH, through the RNA-DNA hybrid, shorter than the distance between the polymerase and RNase H active sites. These truncated substrates can be cleaved by the full-length HIV-1 RT (data not shown), indicating that the cleavages may occur in a polymerase-independent mode for the wild-type HIV-1 RT.

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