We have previously described the in vitro and in vivo characterization of a panel of mutations affecting the RNase H domain of Moloney murine leukemia virus reverse transcriptase (Blain, S. W., and Goff, S. P. (1993) J. Biol. Chem. 268, 23585–23592; Blain, S. W., and Goff, S. P. (1995) J. Virol. 69, 4440–4452). We were intrigued by the discrepancy between in vitro and in vivo RNase H results for two of the mutants. While ΔC and Δ5E appeared to have nearly wild-type RNase H activity in vitro, they were unable to degrade their genomic RNA in vivo and thus were effectively RNase H null mutants in this context. In this present report, we describe the differential effects of these mutations on RNase H activity in vitro in the presence of Mg²⁺ versus Mn²⁺: mutants ΔC and Δ5E were active in the presence of the less biologically relevant Mn²⁺ and not in the presence of Mg²⁺. We also describe three mutants with only partial activity in Mg²⁺. The presence of the different cations can also affect DNA polymerization and processivity of an RNase H-deficient mutant.

Reverse transcriptase (RT)¹ is responsible for converting the single-stranded RNA genome of a retrovirus into double-stranded DNA (1, 2). RT accomplishes this process using two activities: a DNA polymerase activity that is able to synthesize DNA from both RNA and DNA templates and a ribonuclease H activity (RNase H) that is able to degrade RNA present in RNA-DNA hybrid form (for reviews, see Refs. 3 and 4). RNases H release short oligonucleotide products with 5’-PO₄ and 3’-OH groups and show a valley cation requirement for catalysis (for reviews, see Refs. 5–8). The two activities of MMLV RT reside in separable domains: the N-terminal two-thirds of the enzyme contains the DNA polymerase domain, while the RNase H domain is in the C-terminal one-third (9). The RNase H domain of MMLV RT is highly homologous to other RNases H, including Escherichia coli (10, 11) and HIV-1 (12–14) RTs.

Thus, although the structure of MMLV RNase H has not been determined, it is likely that the RNase H domain of this enzyme will be similar to those of other RNases H (15).

RNase H activity has been implicated in several steps in reverse transcription: the enzyme is essential for the viral life cycle, and mutant viruses that lack RNase H activity are noninfectious (16). RT initiates DNA synthesis from a tRNA primer bound to a region near the 5’-end of the genomic RNA termed the primer-binding site (PBS). Elongation of this tRNA to the 5’-end of the genome results in formation of (−)-strand strong stop DNA, the first DNA intermediate to appear during reverse transcription (17). The newly synthesized (−)-strand strong stop DNA forms an RNA-DNA hybrid with the (+)-strand genomic RNA, which is then degraded to permit translocation to the 3’-end of the RNA. Analysis of abortive replication products produced by virions that lack RNase H has shown that the (−)-strand strong stop DNA remains in hybrid form with the genomic RNA, accounting for the observed reduction in translocation and elongation for these mutants (16). In addition to degradation of the genomic RNA, RNase H performs several specialized functions at later times, including the creation and removal of the polypurine tract primer and the removal of the (−)-strand tRNA primer (18).

We previously described the in vitro characterization of a panel of mutations made in the RNase H domain of MMLV RT (19). The design of these mutant enzymes was based on sequence alignments and the crystal structures of E. coli and HIV-1 RNases H and the predicted structure of the MMLV RNase H domain (10–15). Most of the RNase H mutants analyzed retained full or at least partial RNase H activity in vitro as assayed by in situ gel techniques. We additionally characterized these mutants in vivo in the context of the full-length retroviral provirus (20). Two mutants, Δ5E and ΔC RTs, which appeared to retain significant RNase H activity in vitro (50 and 100% activity, respectively), were completely noninfectious as virus in vivo. These mutant viruses were further analyzed in the endogenous assay, in which reverse transcription is carried out in vitro in purified virions in the presence of radiolabeled dNTPs, and various radiolabeled DNA products can be detected. This analysis demonstrated that Δ5E and ΔC left their (−)-strand strong stop DNA in hybrid form with the genomic RNA and thus were effectively RNase H null mutants in this context of the endogenous reaction.

We were intrigued by the discrepancy between the presence of RNase H activity in the in situ gel assay and the absence of RNase H activity in the endogenous assay. We hypothesized that the differential activity detected in these two assays might result from the following: 1) a difference between the recombinant and virion-associated RTs analyzed, 2) a difference between the substrates degraded in these two assays (random heteropolymeric radiolabeled RNA-DNA hybrid in the in situ gel assay versus genomic RNA hybridized to the newly synthe-
sized (−)-strand DNA during the endogenous reaction), 3) the presence of other viral proteins that might affect RT activity during reverse transcription, or 4) a difference between the different cations used in these two assays (Mn$^{2+}$ versus Mg$^{2+}$).

Tests revealed that the basis for the difference in the activity of these mutant enzymes in vitro and in vivo resulted from differential RNase H activity when assayed in the presence of Mg$^{2+}$ or Mn$^{2+}$. In particular, we describe two mutants that appear to be active only in the presence of the less biologically relevant Mn$^{2+}$. The presence of the different cations also appears to affect DNA polymerization and processivity.

**EXPERIMENTAL PROCEDURES**

Description of Mutants—MMLV RTs were constructed by oligonucleotide-mediated site-directed mutagenesis as described previously (19). R6575, Y598B, S526A, Y586F, and D524N contain point mutations in the RNase H domain of MMLV RT; the mutant enzymes were named by appending the amino acid present in wild-type RT, the residue number, and the amino acid present in the mutant at that position. Δ5E has a 5-amino acid deletion from Ser-643 to Arg-647. ΔC has an 11-amino acid deletion from Ile-593 through Leu-603 in the RNase H domain (19, 21). H7 is a linker insertion mutant, containing a frameshift at the start of the RNase H domain, and thus is effectively an RNase H null form of RT (9, 20).

pRT30-2 (the wild-type enzyme), D524N, Δ5E, and ΔC were analyzed as E. coli-expressed proteins, purified as described previously (19, 21). Mutants R6575, Y598B, S526A, Y586F, Δ5E, and H7 were analyzed as virion-associated RTs. To prepare the mutant RTs, these mutations were moved into the context of the full-length provirus pNCA (16, 20). Stable producer lines were generated for these mutant proviruses as described with E. coli's modified Eagle's medium supplemented with 10% NuSerum (Collaborative Research) (19, 20). Maintenance of cells in Dulbecco's modified Eagle's medium supplemented with 10% NuSerum (Collaborative Research) (19, 20). To prepare virions, producer cells were fed Dulbecco's modified Eagle's medium supplemented with 10% NuSerum (Collaborative Research) (19, 20) for 12 h prior to harvest. The virions were pelleted for 3 h at 25,000 rpm, resuspended, layered over a 25/45% sucrose step gradient, and sedimented to the interface. The viral band was collected and repelleted for 2 h at 25,000 rpm following dilution in TNE buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). Viral RTs were assayed without further purification; virions were lysed in Nonidet P-40-buffer containing formamide and fully denatured (90°C for 10 min). The samples were analyzed by electrophoresis on 8% nondecreasing polyacrylamide gels.

**RESULTS**

**RNase H Activity in Mg$^{2+}$ and Mn$^{2+}$: Defined Substrate Assay**—To test for the activity of mutant RTs in vitro in a setting resembling the natural one in the virion, we assayed the mutants using a defined RNA-DNA hybrid as substrate. This substrate consists of a heteropolymeric hybrid with unlabeled (−)-strand RNA spanning the 5' end of the MMLV genome annealed to radiolabeled complementary (−)-strand DNA. This substrate contains the PBS, US, and part of the U3 region and corresponds to the authentic sequence that U3 RNase H encounters during reverse transcription. The assay differs from more conventional solution assays in that we radiolabeled the DNA strand and analyzed the products on an 8% nondenaturing polyacrylamide gel (Fig. 1). The double-stranded hybrid migrates at ~240 base pairs (Fig. 1, lane 19), while the single-stranded DNA species produced upon denaturation of the substrate or RNase H degradation of the RNA migrates more slowly (lane 20). With this substrate, we were able to test both detergent-permeabilized virion-associated RNases H and purified recombinant enzymes. Several of the mutants were analyzed as virion-associated enzymes to avoid possible E. coli nuclease contamination.

Purified recombinant RTs were added to this substrate and incubated at 37°C for 1 h (Fig. 1). Purified E. coli RNase H was able to degrade this substrate efficiently, producing the single-stranded slower species, which corresponded to full RNA degradation and/or release (Fig. 1, lane 2). Purified wild-type RT (pRT30-2) was able to degrade the substrate efficiently in both Mn$^{2+}$ and Mg$^{2+}$ (Fig. 1, lanes 3–8; and Fig. 2, lanes 3–6). However, wild-type RT was ~8–16-fold more active in the presence of Mn$^{2+}$ compared with Mg$^{2+}$ (Fig. 1, compare lanes 6 and 7 in Mn$^{2+}$ to lanes 3 and 4 in Mg$^{2+}$).

Virion-associated wild-type RT was compared with the recombinant enzyme. Wild-type RT from virions was able to...
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Fig. 1. RNase H activity of recombinant proteins in the defined substrate assay. Purified recombinant enzymes were incubated as described under "Experimental Procedures" in either 6 mM MgCl2 or 2 mM MnCl2. Lane 1, TNE buffer control; lane 2, 1 unit of E. coli RNase H (RH) in Mg2+; lanes 3–5, wild-type (WT) RT in Mg2+; lanes 6–8, wild-type RT in Mn2+; lanes 9–11, Δ5E in Mn2+; lanes 12–14, ΔC in Mn2+; lane 15, DS24N in Mg2+; lanes 16–18, DS24N in Mn2+; lane 19, untreated substrate; lane 20, denatured substrate. The RT preparations were normalized with respect to DNA polymerase activity in oligo(dT)poly(rA) primer template assays in Mn2+ as well as by Western blot analysis to compare protein levels. Thus, the 1:32 dilution in lane 5 corresponds to the same amount of protein in lanes 7, 10, 13, and 18. Enzymes were diluted in TNE buffer: ds, double-stranded; ss, single-stranded. Sizes of marker DNAs are indicated to the right.

Fig. 2. RNase H activity of recombinant proteins in the defined substrate assay in Mg2+. Purified recombinant enzymes were treated as described under "Experimental Procedures." All of the enzymes in this panel were analyzed in the presence of Mg2+. Lane 1, E. coli RNase H; lanes 3–6, wild-type (WT) RT; lanes 7–10, Δ5E; lanes 11–14, ΔC; lane 15, DS24N; lane 16, untreated substrate; lane 17, denatured substrate; lane 18, marker DNAs. The wild-type and mutant preparations can be compared with the dilutions used in Fig. 1. ds, double-stranded; ss, single-stranded.

Fig. 3. RNase H activity of detergent-permeabilized virions in the defined substrate assay in Mn2+. Purified virions were permeabilized and assayed as described under "Experimental Procedures." All of the mutants in this panel were analyzed in the presence of Mn2+. Lane 1, H7; lanes 2–4, wild-type (WT) RT; lanes 5–7, S526A; lanes 8–10, R657S; lanes 11–13, Y598V; lanes 14–16, Δ5E; lane 17, untreated substrate; lane 18, denatured substrate. The dilutions listed for the wild type and mutants were normalized with respect to DNA polymerase activity in oligo(dT)poly(rA) primer template assays and by Western blot analysis. It should be noted that the wild-type and mutant preparations are not the same as the recombinant preparations in Figs. 1 and 2.

Fig. 4. RNase H activity of detergent-permeabilized virions in the defined substrate assay in Mg2+. Purified virions were permeabilized as described under "Experimental Procedures." All of the mutants in this panel were analyzed in the presence of Mg2+. Lane 1, E. coli RNase H; lanes 3–6, wild-type (WT) RT; lanes 7–10, S526A; lanes 11–14, R657S; lanes 15–18, Y598V. The wild-type and mutant preparations can be compared with those used in Fig. 3. ds, double-stranded; ss, single-stranded.

in the defined substrate assay to determine if their alterations could differentially affect the cation preferences of the enzymes. Mutants Δ5E and ΔC prepared as recombinant proteins were able to degrade the hybrid efficiently in Mn2+ (Fig. 1, lanes 9–14) almost as well as the wild type (compare lanes 6, 9, and 12). This result is consistent with our previous results in Mn2+ in the in situ gel assay (Table I) (19). In Mg2+, however, these two mutants showed dramatically reduced activity and were unable to completely degrade all of the RNA to release the single-stranded DNA (Fig. 2, lanes 7–14). Some activity was apparent at the highest concentrations of Δ5E (Fig. 2, lane 7); the hybrid form disappeared, but only a slower migrating smear appeared for Δ5E at the 1:1 concentration. This conclu-
**TABLE I**

| Analysis of RNase H mutants |
|----------------------------|
| The in vitro RNase H activities are expressed as a percent of wild-type activity. The values for RNase H activity in the in situ gel assay were determined previously (19). The values for RNase H activity in the defined substrate assay are as follows: wt, wild-type degradation of substrate; 5% low but detectable activity, 25% intermediate degradation species detected. The nature of the (−) strand strong stop DNA (−ss) in the endogenous reverse transcription reaction was determined previously (20). “Hybrid” corresponds to (−) strand strong stop DNA in hybrid form with the genomic RNA, and “ss” corresponds to single-strand (−ss) strong stop DNA. |
| In vitro RNase H activity | Nature of −ss |
|---------------------------|--------------|
| In situ gel, RNase-H defined substrate | Mn^{2+} | Mg^{2+} | −ss |
| D524N | 10–25% | — | Hybrid |
| Y586F | 5% | 5% | Hybrid |
| Δ5E | 50% | wt | — | Hybrid |
| ΔC | wt | wt | — | Hybrid |
| H7 | — | — | — | Hybrid |
| R657S | wt | wt | * | ss |
| S526A | 25% | wt | * | ss |
| Y598V | wt | wt | * | ss |

**Fig. 5.** RNase H activity of detergent-permeabilized virions in the defined substrate assay. Purified virions were permeabilized and assayed as described under “Experimental Procedures.” Lanes 1–4, Δ5E in Mg^{2+}; lanes 5–7, Y586F in Mg^{2+}; lanes 8 and 9, wild-type (WT) RT in Mg^{2+}; lanes 10 and 11, Y586F in Mn^{2+}; lanes 12, 17, and 14, untreated substrate; lane 15, denatured substrate. The dilutions listed for the wild type and mutants can be compared with the dilutions used in Figs. 3 and 4. ds, double-stranded; ss, single-stranded.

pH, since these enzymes were purified from *E. coli*, we could not rule out the possibility that the residual activity of Δ5E and ΔC at higher enzyme concentrations was due to contaminating *E. coli* RNase H activities, although by in situ gel techniques, these enzyme preparations did not contain any other detectable RNase H activities (19, 21). To address this concern, Δ5E was analyzed as a virion-associated enzyme. Δ5E exhibited wild-type activity in Mn^{2+}, but little or no activity in Mg^{2+} (Fig. 3, lanes 14–16; and Fig. 5, lanes 1–4). As a virion-associated enzyme, the slower migrating smear was not detected, even at high enzyme concentrations (Fig. 5, lanes 1–4). Thus, Δ5E behaved effectively as an RNase H null mutant in Mg^{2+}. This result might account for the discrepancy in the activities seen in the endogenous reaction and the in situ gel assay for mutants Δ5E and ΔC (Table I). While Δ5E and ΔC do retain nearly wild-type RNA-DNA nuclease activity in Mn^{2+}, they are essentially inactive in the presence of Mg^{2+}.

Several other mutants were analyzed to determine whether the differential ability to degrade RNA depending on the divalent cation used was a common feature of many RNase H mutants. Mutant D524N, containing a change in a residue implicated in catalytic activity, was unable to degrade the defined substrate efficiently in either Mg^{2+} or Mn^{2+} (Fig. 1, lanes 15–18; and Fig. 2, lane 15). Even when almost 16-fold more D524N enzyme was analyzed, no activity on the defined substrate was observed in the presence of Mn^{2+} (Fig. 1, compare lane 16 for D524N with lane 7 for the wild type). Likewise, mutant Y586F, assayed as a virion-associated enzyme, was almost completely inactive in Mg^{2+} and Mn^{2+} (Fig. 5, lanes 5–7, 10, and 11). At a 1:128 dilution, where the wild type was able to degrade the substrate efficiently, Y586F was completely inactive (Fig. 5, lane 11); at a 1:32 dilution, the mutant was slightly active in Mn^{2+} (lane 10). As mutant Y586F has a tendency to revert to a more active RNase H form during cell culture (24), this slight activity may be the result of trace amounts of this reverted enzyme contaminating this viral preparation. It is clear that D524N and Y586F were essentially inactive RNases H in the presence of both cations. These results suggest that the differential activity of Δ5E and ΔC in the presence of Mg^{2+} is a defect specific to these two, and not a phenotype intrinsic to all RNase H mutants.

Mutants S526A, R657S, and Y598V were previously shown to be active in the in situ assay and are able to degrade their genomic RNA as well as the wild type in the endogenous reaction (Table I) (19, 20). When these mutants were analyzed as virion-associated RTs on the defined substrate, all had wild-type activity in Mn^{2+} (Fig. 3, lanes 5–13). In the presence of Mg^{2+}, however, only partial activity was observed, and a novel intermediate degradation species was detected (Fig. 4, lanes 7–18). The double-stranded substrate migrating at 240 base pairs disappeared when the mutants were assayed at the 1:8 enzyme concentration, but instead of releasing the single-stranded product corresponding to complete RNA digestion, these mutants appeared to chase much of the hybrid to a form that migrated only slightly more slowly (Fig. 4, compare lane 4 for the wild type to lanes 9, 13, and 17 for the mutants). Mutant Δ5E left the substrate completely double-stranded at this 1:8 concentration and at higher concentrations, ruling out the possibility that this was due to a contaminating background activity (Fig. 5, lanes 1–4). We suspect that this form corresponds to a discrete DNA-RNA species, but cannot estimate how much RNA remains associated with the DNA. S526A did degrade the substrate to the fully single-stranded form when increasing amounts of enzyme were assayed (Fig. 4, lane 7). The intermediate form can also be seen for the wild type at very low enzyme concentrations (Fig. 4, lane 6). These results suggest that while these mutants are active RNases H, they may be less effective than wild-type RT at removing all the RNA from these substrates in Mg^{2+}.

**Rescue of DNA Polymerase Activity of Mutant H7 by Mn^{2+}—**

RNase H mutants of RT analyzed in the endogenous reverse transcription assay frequently show defects in DNA elongation (16, 20, 21). The endogenous assay, typically performed in Mg^{2+}, permits the detection of various DNA intermediates using, as a template, the endogenous viral RNA in permeabilized virion particles. (−) Strand strong stop DNA is readily detected in reactions with wild-type virions, and some RNase H mutants cannot even complete synthesis of this DNA (20).
Treatment of the endogenous reaction products with RNase A in high salt will only degrade single-stranded RNA, leaving intact single- or double-stranded form. Treatment with RNase A in low salt (Fig. 6, lane 7) produced predominantly a truncated form of (-)-strand strong stop DNA, termed VFF for "very fast form," in the endogenous reaction in Mg\(^{2+}\) (Fig. 6) (20, 21, 25). Little or no completed full-length DNA was detected after RNase A treatment in high salt (Fig. 6, lane 1). When the VFF species was denatured, very little of the 163-nucleotide product was detected, confirming the tendency of this mutant to pause prematurely (Fig. 6, lane 3). However, assays using the other cation showed that mutant H7 was indeed able to synthesize full-length (-)-strand strong stop DNA in Mn\(^{2+}\). Treatment of the Mn\(^{2+}\) reaction products with RNase A in high salt produced the FF species (Fig. 6, lane 4); after denaturation, the 163-nucleotide form was detected (lane 6). Treatment with RNase A in low salt produced the 145-nucleotide form (Fig. 6, lane 5). Thus, the DNA polymerase activity of the mutant was enhanced in Mn\(^{2+}\) such that efficient formation of the (-)-strand strong stop DNA was induced. The signature of this DNA, the FF species, was in double-stranded form. Thus, although Mn\(^{2+}\) improved the ability of the DNA polymerase to synthesize the full-length strong stop DNA, it could not restore RNase H activity to this null mutant.

**DISCUSSION**

Previous analyses indicated that the mutant RTs \(\Delta 5E\) and \(\Delta C\) had considerable RNase H activity in vitro as assayed by in situ gel techniques, but left the (-)-strand strong stop DNA in hybrid form in the endogenous reaction. The experiments described here show that \(\Delta C\) and \(\Delta 5E\) could efficiently degrade an RNA-DNA substrate with Mn\(^{2+}\) but not with Mg\(^{2+}\) as divalent cation (Table I). As Mg\(^{2+}\) is probably the biologically relevant divalent cation, these two mutants are effectively RNase H null mutants in vivo, consistent with the observed loss of infectivity and reduction in strand translocation in the mutant viruses (20).

The effects seen in these two mutants are more extreme versions of effects seen with other enzymes and mutants. In the defined substrate assay, wild-type MMLV RT RNase H was ~16-fold more active in the presence of Mn\(^{2+}\) compared with Mg\(^{2+}\), assayed both as a purified recombinant protein and as a detergent-permeabilized virion-associated enzyme. Differential activities with the two cations have been observed for other RNases H in other assays as well. The HIV-1 RNase H single domain, expressed as a hexahistidine-tagged fusion protein independently of the DNA polymerase domain, similarly exhibits its Mn\(^{2+}\)-but not Mg\(^{2+}\)-dependent RNase H activity (26). Furthermore, the addition of an appropriate C-helix into an inactive single-domain version of the HIV-1 RNase H, which normally lacks this helix, restores Mn\(^{2+}\)-but not Mg\(^{2+}\)-dependent activity (27, 28). However, it should be noted that E. coli RNase H and HIV-1 RNase H as assayed in the intact RT prefer Mg\(^{2+}\) for optimal catalysis.

Analogous divalent-dependent behavior has been reported for many nucleic acid-binding enzymes (e.g. Refs. 29 and 30; very recently, Ref. 31). The basis for the differential activity of these enzymes with the two divalent cations is unclear, but could be due to aspects of the cation binding with the enzyme or with the substrate. Determination of cation requirements is

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**Fig. 6.** RNase A treatment in high and low salt of endogenous reaction products synthesized in Mn\(^{2+}\). The endogenous reaction was performed as described under "Experimental Procedures." Lanes 1–6, H7; lanes 7–9, \(\Delta 5E\). Lanes 1–3 and 7–9 were performed in the presence of 6 mM MgCl\(_2\). Lanes 4–6 were performed in the presence of 2 mM MnCl\(_2\). The endogenous reaction products were treated with RNase A in high (H; lanes 1, 3, 4, 6, 7, and 9) or low (L; lanes 2, 5, and 8) salt buffer prior to electrophoresis on 8% nondenaturing polyacrylamide gels. Lanes 2, 3, 5, 6, 8, and 9 were denatured prior to loading by suspension in dye-containing formamide. Lanes 1, 4, and 7 were analyzed without denaturation. The VFF and FF forms are labeled. nt, nucleotide.
complicated by the fact that they bind not only to enzymes, but also to nucleic acids and dNTPs, leading to different template and substrate complexes depending on the cation used (32).

There is some evidence that these mutant enzymes, assayed in Mg$^{2+}$, may be affected in substrate binding. It is interesting to note that the ΔC mutation removes a basic handle region that has been implicated in preferential substrate binding (21, 33). Mutation and substitution of the lysines in this region in the E. coli RNase H enzyme raise the $K_m$ without changing the $V_{max}$ (33). However, the ΔSE mutation removes a loop between the fifth $\beta$-sheet and the last $\alpha$-helix, near a conserved histidine residue that has been implicated in catalysis rather than substrate binding (34, 35).

Mn$^{2+}$ and Mg$^{2+}$ may bind to RNase H in two different positions. While the crystal structures of several RNases H are known, it remains unclear whether one or two divalent cations are involved in catalysis. A two-cation mechanism, similar to that for the 3' → 5' exonuclease of DNA polymerase I, has been suggested for E. coli RNase H (10, 36). Additionally, crystallographic analysis of the HIV-1 RNase H single domain revealed two divalent cations (Mn$^{2+}$) bound at the enzyme's active site (12). But in other studies, a single Mg$^{2+}$ was observed bound at the active site of E. coli RNase H (11, 37). As all of these structures were determined in the absence of substrate, it is difficult to ascertain whether the observed cation binding is productive or rather inappropriate binding that would be altered when the substrate was present. Co-crystallization of RNase H with an RNA-DNA substrate would help address this issue.

We also detected significant differences between RNase H activities measured in the in situ gel assay and the defined substrate assay even when both assays were carried out with Mn$^{2+}$. D524N had −10% activity in the in situ gel assay, but was inactive in the defined substrate assay, and conversely, S526A was fully active in the defined substrate assay, but had only 25% activity in the in situ assay. During the in situ assay, the gel is typically allowed to renature for several days, during which time the enzyme is constantly surrounded by substrate. Thus, this assay may not be sensitive to subtle defects in affinity. An additional difference that may account for the discrepancies between the two assays is the fact that proteins must renature in the in situ gel assay, and mutations may specifically affect this step.

With amounts of enzyme sufficient for wild-type RT to fully degrade the RNA from the radiolabeled DNA substrate in the RNase H defined substrate reaction, mutants S526A, R657S, and Y598V showed some activity in Mg$^{2+}$, but were unable to degrade all of the RNA from the substrate. The full-length double-stranded species completely disappeared, and a new discrete species was detected. While we cannot tell how much RNA was removed by these mutants, the major product was the same for all three, and at low concentrations, this product was detected with wild-type RT. This result suggests that these mutants may be less processive RNases H than the wild type or that they only cleaved efficiently up to a specific site in the RNA. The structural features of the substrate that might determine the accumulation of this intermediate are unknown. It should be noted that this cleavage is not due to background activity seen for all the mutants: similar products were not seen with mutant ΔSE, H7, or Y586F. The defects seen in this assay may be among those responsible for the delayed replication of viruses carrying these mutations (20).

In the endogenous reaction, mutants S526A, R657S, and Y598V were able to degrade their genomic RNA completely, like wild-type RT. What then is the difference between the endogenous reaction and the RNase H defined substrate reaction? One main difference is the presence of a more intact capsid in the endogenous reaction and the resulting increased effective concentration of other viral proteins. Virions were permeabilized with 0.01% Nonidet P-40 in the endogenous reaction and were more fully lysed with 0.3% Nonidet P-40 in the in vitro defined substrate reactions. Thus, processive RNase H activity may be affected by the presence of other retroviral proteins (most notably NC, the basic single-strand nucleic acid-binding protein), which are diluted out during the defined substrate reaction. Although we do not favor the idea, we cannot rule out the possibility that the mutant enzymes are simply more sensitive to high Nonidet P-40 concentrations.

A second significant difference between the two assays is the fact that DNA polymerization and RNA degradation may occur simultaneously during the endogenous reaction. It is possible that these mutants are more active in degradation when it is coupled to polymerization, a feature that we are not testing during the defined substrate reaction. There is evidence that RNase H may behave differently when coupled with polymerization (38, 39). Further analysis of these mutations may help us understand their differential effects in the endogenous and defined substrate assays, in an attempt to understand polymerization-dependent and -independent RNase H activity.

Previous work has shown that the DNA polymerase activity of most RTs, including HIV-1, Rous sarcoma virus, and MMLV RTs, prefers Mg$^{2+}$ for full activity. The DNA polymerase activity of MMLV RT is unusual in preferring Mn$^{2+}$ over Mg$^{2+}$ as a divalent cation (40–43). MMLV RT shows a reduced rate of synthesis in Mg$^{2+}$, and the DNA products are also generally shorter, suggesting that the enzyme may be less processive (Ref. 40 and data not shown). Mutations in the RNase H domain can also affect the DNA polymerase activity and can particularly reduce its ability to form long products (25). In these studies, we found that Mn$^{2+}$ is able to qualitatively influence DNA polymerase processivity during the endogenous reaction. In the presence of MnCl$_2$, the normally nonprocessive mutant H7 was converted to a more processive form, efficiently synthesizing full-length (→) strand strong stop DNA. Thus, assays of MMLV RT in Mn$^{2+}$ show alterations in both the DNA polymerase and RNase H activities, generally showing enhanced activity and masking significant defects. These results suggest that RT should be assayed in Mg$^{2+}$ to detect biologically significant effects with the greatest sensitivity.

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