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# Mutational analysis of the reverse transcriptase and ribonuclease H domains of the human foamy virus

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## ABSTRACT

**Human foamy or spuma virus (HFV) codes for a distinct set of *pol* gene products. To determine the minimal requirements for the HFV enzymatic activities, defined residues of the reverse transcriptase (RT) and ribonuclease H (RNase H) domain of the HFV *pol* gene were mutated by site-specific PCR mutagenesis. The mutant gene products were bacterially expressed, purified by $Ni^{2+}$ chelate affinity chromatography and characterised by Western blotting. The enzymatic activities of the individual recombinant HFV *pol* mutant proteins were characterised by *in situ* RT, RNase H and RNase H* assays. Two substitution mutants reached RT activity levels higher than that of the intact recombinant HFV RT-RH-His. When the catalytically essential D508 was substituted by A508, 5% of RNase H activity was retained while DNA polymerase activity increased 2-fold. A deletion of 11 amino acid residues in the hinge region completely abolished DNA polymerase while RNase H activity decreased 2-fold. A deletion mutant in the C-terminal RH domain showed no RNase H but retained RNase H* activity indicating that the activities are genetically separable. The combined data reveal that the HFV DNA polymerase and RNase H activities are interdependent.**

## INTRODUCTION

Spuma or foamy viruses have some features that set them apart from other retroviruses. Recently, the main attention has been focused on the *bel* genes and on gene expression by an internal non-LTR promoter (15). Although the classical *gag, pol* and *env* genes of foamy viruses are also clearly distinct in sequences, their phenotypic properties have not been studied. The foamy viral *propol* gene codes for the proteinase, the DNA polymerase, the ribonuclease H (RH) and the integrase (IN) domains (for reviews see 6,16,17). Since HFV is strongly cell-associated which renders the availability of *in vivo* synthesised viral proteins difficult, it is of great advantage to use bacterial expression of HFV recombinants as means to study biochemical properties of the HFV enzymes. After expression of the HFV genes in *Escherichia coli* and subsequent purification by $Ni^{2+}$-chelate chromatography, the His-tagged recombinant proteins were shown to be enzymatically active either as DNA polymerase, ribonuclease H or as integrase (13,21). The properties of the corresponding enzymes of HIV-1 and MLV were reported from several laboratories (9,4,12,19, 23,26).

Immunodetection with HFV RNase H-specific antiserum revealed *pol* proteins of 120 and 80 kDa in wild-type-infected cells. In addition, another active *pol* protein of 60 kDa was detectable by *in situ* reverse transcriptase (RT) gel assays. The immature 120 kDa *pol* precursor carries the integrase domain of 39 kDa (13). It is likely that the *pol* precursor is proteolytically processed to the 80 kDa and, in addition, also to the 60 kDa proteins that lacks the RH domain. In *in situ* gel RT assays, both the mature 80 and the 60 kDa *pol* proteins had much higher DNA polymerase activities than the 120 kDa *pol* precursor. Although an active RH domain can be expressed as a separate protein independent of the DNA polymerase activity, it remains unclear whether the two domains are interdependent *in vivo* (for review, see 12). In the presence of $Mn^{2+}$, some retroviral *pol* gene products in addition to specifically degrading the RNA part of a DNA–RNA hybrid were recently shown to degrade double-stranded RNA (10); this activity was named RNase H*. The *in vivo* role of any retroviral RNase H* remains doubtful.

Our current interest is focussed on the minimal requirements of both enzymatic activities and on the mutual interdependence of the DNA polymerase and RNase H activities of HFV RT. To approach these questions, we used PCR-mutagenesis and analysis of the enzymatic activities of the corresponding mutated and purified recombinant HFV proteins. We are interested to study the known protein sequence differences between the various known retroviral enzymes and those of HFV in order to define more precisely the corresponding domains and to develop a rationale for producing HFV proteins with higher specific activities, since the wild-type HFV activities are relative low compared to those of other retroviral enzymes.

## MATERIALS AND METHODS

Cloning of HFV RT mutants. Clone pET22bRT1 (13,25) was used as starting material for the construction of seven different novel HFV *pol* mutants. Oligonucleotide-mediated site-directed mutagenesis was by polymerase chain reaction (PCR) employed

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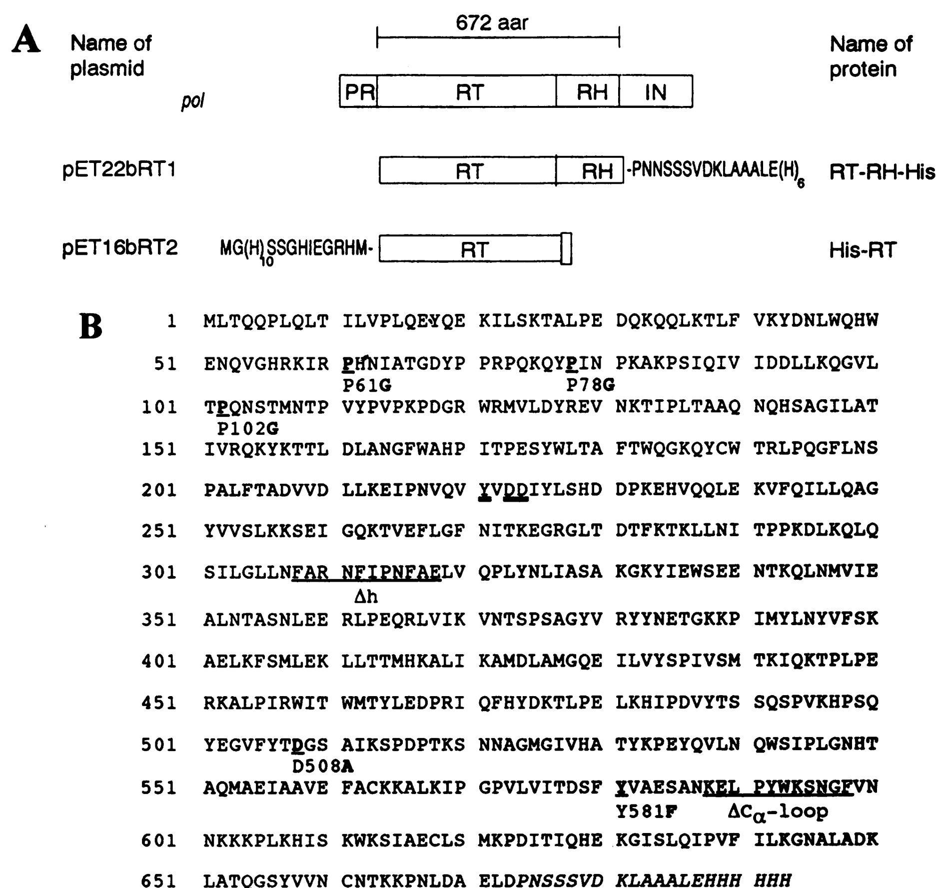


**Figure 1.** (**A**) Schematic diagram of two recombinant His-tagged HFV reverse transcriptase and RNAse H clones (RT-RH-His and His-RT). (**B**) The corresponding primary structure of RT-RH-His. Recombinant clone pET22bRT1 (RT-RH-His) was constructed as reported previously (13). The substitution and deletion mutants analysed were generated by PCR with the appropriate primers as described in Materials and Methods. The locations of the individual mutants are shown below the wild-type HFV RT sequence, the position of replaced amino acid residue is underlined; the mutated residue is in bold. The two deletion mutants, Δh located in the tether region, and $\Delta c_{\alpha}$-loop in the RH domain are underlined. The YM/VDD box conserved in all known retroviral *pol* sequences that has been shown to be part of the catalytic center of the polymerase is doubly underlined. The C-terminal sequences of RT-RH-His derived from the vector pET22b is shown in italics. Recombinant clone His-RT carries the His-tag at its N-terminus.

to introduce the individual mutations. XBAS (5′-TACGACTCA-CTATAGGGG-3′) and AVAAS (5′-GGCTTTGTTAGCAGCC-GG-3′) served as oligodeoxynucleotide primers for regions adjacent to the mutation sites. For PCR amplifications, P61S (5′-AAAATTCG*TGG*CCATAATATAGCAACTGT-3′) and P61GAS (5′-ATTATGGCCACGAATTTTTCTATGCGAC-3′) served as primers for mutant P61G; primer pair P78GS (5′-*GGT*ATTAATCCCAAGGCAAAGCCTAGT-3′) and P78G AS (5′-CTTGGGATTAATA*CC*ATATTGTTTTTGAGG-3′) for P78G; P102GS (5′-AC*TGG*CCAAAATAGTACAATGAATCA-CCA-3′) and P102GAS (5′-ACTATTTTGG*CCA*GTTACACC-CCTTGTTT-3′) for P102G; D508AS (5′-*GCC*GGC TC-GGCCATCAAAAGTCCT-3′) and D508AAS (5′-GATGGCG-AGCC*GGC*AGTATAAAACACTCC-3′) for D508A; Y581FS (5′-*TTC*GTAGCCGAATCGGCTAATAAAGAATTACCA-3′) and Y581FAS (5′-CGATTCGGCTAC*GAA*GAAATATCAGT-TATAAC-3′) for Y581F; DHS (5′-AAGCAATTACA*ATCGAT*-ACTGGTACAACCATTA-3′) and DHAS (5′-TA*TCGAT*TGT-AATTGCTTTAAGTCTTTTGG-3′); for Δh; DcLOOPS (5′-CC-ATACTGGAAATC*GAT*TCCAAATGGAAATCT-3′), and DcLOOPAS (5′-CGATTTCCAGTATGGTAATTC-3′) was used for the construction of the deletion of the $\Delta c_{\alpha}$-loop in the RH region. Mutated bases are in italics, restriction enzyme cleavage sites are underlined. The amplified PCR reactions products were cut with *Xba*I and *Ava*I and purified. In parallel, the vector backbone of plasmid pET22bRT1 was digested with the same pair of restriction enzymes and gel purified. To transfer the mutations separately into the *E.coli* expression plasmid, both components were ligated with T4 DNA ligase. *E.coli* BL21(DE3)-cells were subsequently transformed. All mutated constructs were confirmed by restriction enzyme analysis and dideoxy sequencing.

Purification or recombinant mutant proteins and *in situ* RT, RH and RH* gel assays. The purification of the HFV *pol* mutated proteins under denaturing conditions and the $Ni^{2+}$ column affinity chromatography was carried out as described previously (13). The *in situ* gel assays were done as according to (2,7,13). Quantitations of the *in situ* RT activities were carried out with a PhosphoImager (Molecular Dynamics). Western blotting analysis was performed as previously described (14).

For RT assays, RT-RH-His or His-ΔRT-RH, 20 pmol each, were incubated with 1.0 μg poly rA:oligo $(dT)_{12-16}$ and 0.2 μl [$\alpha$-$^{32}$P]dTTP, specific activity 3000 Ci/mmol, in buffer A in a final volume of 100 μl. Buffer A: 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM $MnCl_2$, 10 mM DTT, 10 μM non-radioactive dTTP,

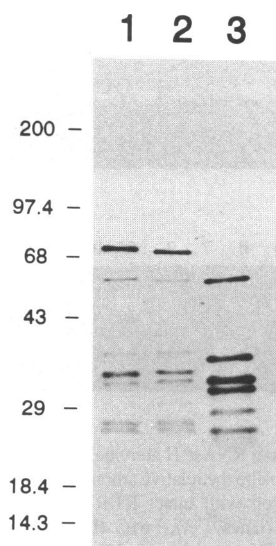


**Figure 2.** Immunodetection of purified HFV *pol* deletion mutant proteins. Recombinant RT-RH-His (lane 1), mutated Δh (lane 2) and $\Delta c_{\alpha}$-loop protein (lane 3) were reacted separately with an RNAse H-specific antiserum. For the location of the deletions see Figure 1B. Numbers on the left margin indicate the positions of molecular size markers in kDa.

0.5 U/μl RNAsin, 100 μg/ml BSA and 0.05% NP-40. Aliquots were taken at the time points indicated.

## RESULTS

The centrally located polymerase and RNase H domains of the HFV *pol* gene shown in Figure 1A as part of recombinant His-tagged proteins have been reported to be active as DNA polymerase, RNAse H and as RNAse H* (13). To test the influence of two classes of defined mutations on the enzymatic activities, we utilized oligonucleotide-mediated site-directed mutagenesis by PCR to introduce the mutations into either the DNA polymerase or the RNase H domain. As rationale for mutating an individual amino acid residue (aar), we used information on a number of well-studied murine leukemia virus (MLV) and HIV-1 *pol* mutants reported previously (2–4,18). In addition, the available three-dimensional structures of HIV-1 RT (11) and of *E.coli* RNAse H were employed as guidelines to select the mutations indicated in Figure 1B. The point mutations selected were expected to minimise overall structural changes and should be helpful to gain insight into the interdependence of RT and RH activities of the HFV *pol* domain.

Two mutants, the $\Delta c_{\alpha}$-loop and the Δh mutants both of which had deletions of 11 aar in the RH loop C or in the hinge region between the polymerase and the RNase H domain, respectively (Fig. 1B), were bacterially expressed and purified by affinity column chromatography. The stabilities of the mutated gene products were studied by Western blotting analysis using a polyclonal antibody directed against the HFV RH domain and part of the connection or hinge region of the HFV *pol* domain (13). In contrast to the intact RT-RH-His recombinant and the Δh mutant proteins that both migrated at ~72 kDa, the $\Delta c_{\alpha}$-loop protein was barely detectable with this size. Instead, a faster moving band of ~62 kDa was the largest of the more prominent degradation products (Fig. 2). The 62 kDa-species was also observed in the intact recombinant RT-RH-His (lane 1) and all other HFV mutants analysed (Fig. 3).

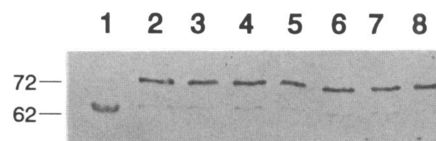


**Figure 3.** Standardization of level of purified HFV RT mutant proteins by Western blotting. $\Delta c_{\alpha}$-loop (lane 1), Δh (lane 2), Y581F (lane 3), D508A (lane 4), P102G (lane 5), P78G (lane 6), P61G (lane 7) and RT-RH-His (lane 8).

Thus deletion of loop C seemed to promote cleavage at a labile site of the mutant protein by *E.coli* proteinases. The degradation products of a given preparation of the mutant proteins were detectable prior to any purification steps. They co-purified and were present during the enzymatic assays performed. Their presence, however, should not interfere with the *in situ* assays, since the activities of the intact proteins of the expected molecular size were separable from the degradation products.

To downregulate the RNase H activity, the Asp 508 residue of the RT-RH-His clone was mutated to Ala (Fig. 1, D508A). The D residue was selected, since it has been reported to be required for the catalytic activity of RNase H from different sources, and conserved among retroviral *pol* sequences. When the purified recombinant D508 mutant protein was tested by *in situ* gel assays, an ~2-fold higher DNA polymerase activity was observed; in contrast, only ~5–10% of the RNase H activity of the intact RT-RH-His protein was retained (Fig. 5; see also Table 1). This result is in agreement with the low RNase H activities reported for mutated MLV and HIV-1 RT proteins (2,18) and implies that the D508 residue is essential for RNase H but not for DNA polymerase activity. When this mutant was assayed for RNase H* activity, it showed low activity (Fig. 6) again comparable with that of the corresponding MLV mutant (2).

A MLV mutant corresponding to Y581F of HFV RH had been reported to retain both DNA polymerase and RNase H* activities but showed only a decreased RNase H activity of 5% (2). In contrast, HFV mutant Y581F had higher than unmutated polymerase and 50% RNase H activity and completely retained RNase H* activity (Table 1). This result indicates that MLV and HFV *pol* enzymatic activities showed distinct activity profiles despite an overall similarity. To study this further, a HFV deletion mutant, $\Delta c_{\alpha}$-loop (Fig. 1) was constructed according to the three-dimensional *E.coli* RH structure and results on MLV RH mutants with a deletion proximal to the C-terminus of the RH domain (2,20,27). This HFV mutant protein had virtually lost polymerase and RNase H activity whereas the major proteolytic cleavage product of the $\Delta c_{\alpha}$-loop mutant retained about one fourth of the RNase H* activity of the intact RT-RH-His protein (Figs 4–6; Table 1).

Close inspection of the connection or hinge regions located between the polymerase and RH domains of different retroviruses has shown that this subdomain varies in length. It is longest in MLV and HFV but relatively short for HIV-1 RT. To examine whether or not there is a strong effect of a region shared by both HFV and MLV, the HFV deletion mutant, Δh, was constructed, purified and assayed for the three enzymatic activities. It was found that the Δh deletion almost abolished polymerase activity while RNase H activity reached 50% and RNase H* 100%, respectively. This result shows that this subregion of the hinge or tether domain is crucial for the RT and RNase H activities while the RNase H* seems to be relative insensitive to changes in the connection region.

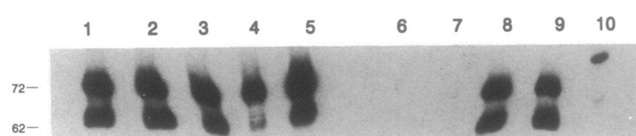


**Figure 4.** Quantitation of RT activities of HFV mutant proteins by *in situ* gel assays. The gels were dried, exposed and the relative amounts of incorporated [α-$^{32}$P]dTTP were determined with a phosphoimager. RT-RH-His (lanes 1 and 9), P61G (lane 2), P78G (lane 3), P102G (lane 4), D508A (lane 5), $\Delta c_{\alpha}$-loop (lane 6), Δh (lane 7), Y581F (lane 8) and pET16b cell extracts as control (lane 10).

**Table 1.** Summary of results of HFV RT, RH and RH* activities

| Mutants[a] | RT[b] | RNAse H[c] | RNAse H*[c] |
|---|---|---|---|
| P61G | 105.3 | 100 | 100 |
| P78G | 71.2 | 100 | 100 |
| P102G | 58.6 | 100 | 100 |
| D508A | 180.3 | 5–10 | 5–10 |
| Y581F | 173.9 | 50 | 100 |
| Δh | 1.4 | 50 | 100 |
| $\Delta c_{\alpha}$-loop | 0.4 | 0 | 25 |

[a]Locations of HFV pol mutants and amino acid substitutions are listed for each mutant in see Figure 1.
[b]RT activities were quantitated with a phosphoimager.
[c]RH and RH* activities were estimated as described in Materials and Methods.

Since the $\Delta c_{\alpha}$-loop mutant produced a C-terminal His-tagged protein that was subsequently purified by affinity chromatography, it is likely that proteolytic degradation of the 62 kDa protein occurred at the $NH_2$- and not at the COOH-end. In agreement with this assumption, it was shown previously that recombinant His-ΔRT-RH that carries an N-terminal truncation was highly active as RNase H (13). In contrast to RT-RH-His that starts at aar 99 of HFV *pol*, His-ΔRT-RH that begins at aar 180 did not have any measurable RT activity in a conventional RT assay (Fig. 7).

Substitution mutants P61G, P78G and P102G (Fig. 1B) were assayed to determine if residues in the N-terminal end of HFV *pol* are essential for DNA polymerase activity. Mutant protein P78G had ~71% RT activity compared with the non-mutated HFV RT-RH-His (Table 1; Figs 2 and 3) and no change in RNase H and H* activities. The corresponding analogous HIV-1 mutant had an RT activity of ~30% (3). The polymerase mutant P102G was reduced to 58% whereas mutant P61G had wild-type levels of polymerase activity (Table 1). The mutants analysed showed unaltered RNase H and H* activities assuming that this part of the RT domain does not influence the RNase H region. The data imply that the N-terminal border between the protease and polymerase domain of HFV *pol* is upstream of the P78 residue (Fig. 1).

## DISCUSSION

We have constructed and examined several HFV *pol* mutants in RNA-dependent DNA polymerase and RNase H activity by using highly purified recombinant His-tagged proteins in an effort to search for mutant forms that display higher enzymatic activities. The mutations were chosen to minimise the effects on folding i.e.

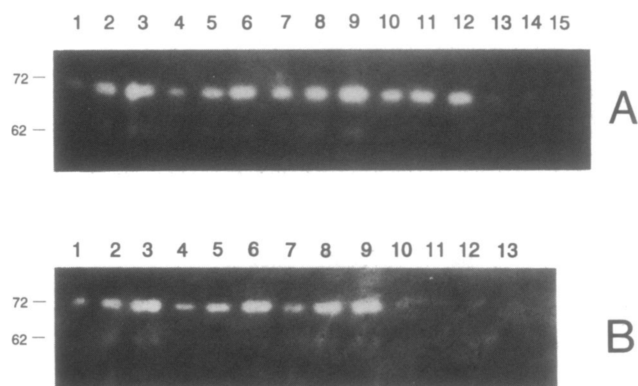


**Figure 5.** Autoradiogram of RNAse H activities of HFV mutant proteins by *in situ* gel assays. After exposure the relative amounts of [$^{32}$P]UMP released were determined by comparison with intact RT-RH-His. (**A**) Successive 2-fold dilutions of RT-RH-His (lanes 1–3), P61G (lanes 4–6), P78G (lanes 7–9), P102G (lanes 10–12) and D508A (lanes 13–15). (**B**) Successive 2-fold dilutions of RT-RH-His (lanes 1–3), Y581F (lanes 4–6), Δh (lanes 7–9), $\Delta c_{\alpha}$-loop (lanes 10–12), and as control cell extracts from parental vector pET16b (lane 13). Numbers on the left indicate molecular size markers in kDa.

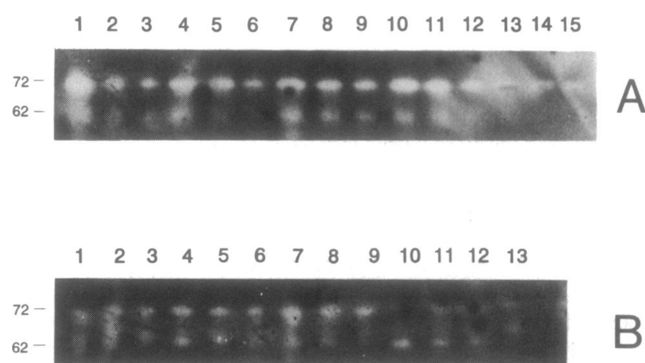


**Figure 6.** Autoradiogram of RNAse H* activities of HFV mutant proteins by *in situ* assays. After exposing the gels, the relative amounts of [$^{32}$P]UMP released were determined by comparison with intact RT-RH-His. (**A**) Successive 2-fold dilutions of RT-RH-His (lanes 1–3), P61G (lanes 4–6), P78G (lanes 7–9), P102G (lanes 10–12), and D508A (lanes 13–15). (**B**) Successive 2-fold dilutions of RT-RH-His (lanes 1–3), Y581F (lanes 4–6), Δh (lanes 7–9), $\Delta c_{\alpha}$-loop (lanes 10–12), and as control cell extracts from parental vector pET16b (lane 13).

the amino acid residues introduced were as similar as possible to the residues substituted. However, it cannot be ruled out that there is great effect of these residues on the renaturation probability of the mutated proteins. The mutated gene products were enzymatically active and compared to the activities of the intact recombinant *pol* proteins. The results of the different assays used showed that mutated residues in the RH domain influenced the associated DNA polymerase activity. The effects were clearly detectable as a higher level of polymerase activity in the RNase H-defective mutants. This is consistent with the increased DNA polymerase activity of recombinant proteins that lack the RH domain (13).

When the lengths of the DNA reaction products were determined, it was found that the sizes of the cDNAs were larger resulting from the reactions with those HFV recombinant enzymes that had a diminished or completely abolished RNase H activity (unpublished observation).

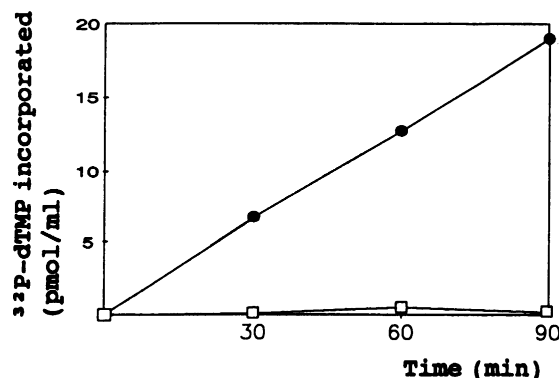


**Figure 7.** Kinetics of HFV RT recombinant proteins. DNA polymerase reactions were carried out as described in Materials and Methods. Non-radioactive dTTP (10 μM) and 20 pmol of purified His-RT (●–●) or His-ΔRT-RH (□–□) were incubated with 1.0 μg poly (rA):oligo (dT) and 2 μl [α-$^{32}$P]dTTP (3000 Ci/mmol) in a final volume of 100 μl. Aliquots were taken at the indicated time points.

The data obtained with the Δh deletion mutant that had virtually lost DNA polymerase but retained 50% of RNase H activity shows that the tether domain is essential for full enzymatic activity of both enzymes. The results suggest that the HFV DNA polymerase and the RNase H domain are interdependent. The three-dimensional structure of the HIV-1 heterodimeric RT reveals that the interaction between the monomers occurs within the connection domain (11). By analogy, this might indicate that the three-dimensional structure of HFV RT has a spatial arrangement slightly different from that of MLV.

Our results also show that the N-terminus of the *pol* domain has to be located upstream of the Pro78, since the point mutation P78G has decreased DNA polymerase activity compared with that of the intact RT-RH-His protein.

Both HFV RNase H deletion mutants exhibited a differential activity pattern with respect to both the RNA/DNA and RNA/RNA nucleases. Some of the RNase H mutants of the MLV RT analyzed by Blain and Goff (2) indicate that the two activities are genetically separable. MLV mutant Y586F, that corresponds precisely to HFV mutant Y581F, showed RNase H* activity similar to the MLV wild-type enzyme but only 5% of MLV RNase H activity. The decrease to 50% of the RNase H activity of the HFV mutant Y581F is consistent with this result. In addition, the enzymatic activity of MLV mutant R675S (2) is comparable with that of the $\Delta c_{\alpha}$-loop mutant of HFV. This HFV mutant was relatively unstable upon bacterial expression. The major proteolytic cleavage product of both the HFV $\Delta c_{\alpha}$-loop and the MLV mutant proteins had RNase H* activity. It has been reported previously that part of the DNA polymerase domain is essential for RNase H* activity, since separately expressed RNase H domains of MLV, HIV-1 and HFV that are active as RNase H did not possess any RNase H* activity (1,2,10,13,24).

Quite recently, the RNase H* of HIV-1 RT was shown to cleave ds RNA in a endonucleolytically manner at a fixed distance of 18 bp from the primer terminus (8). In contrast, the RNA of RNA–DNA hybrids can be degraded to fragments of 7 bp by the synthesis-independent activity (8,22). HFV proteins of His-RT-RH and of the $\Delta c_{\alpha}$-loop mutant were inactive as DNA polymerase but retained RNase H* activity. Although the ds RNA nuclease activity might not have biological relevance, the MLV and HFV data are compatible with the notion that at least part of the active site of RNase H* is distinct or in a different conformation compared with the catalytic site of RNase H.

## ACKNOWLEDGEMENTS

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