Contributions of DNA Polymerase Subdomains to the RNase H Activity of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

JEFFREY S. SMITH, KIRA GRITSMAN, AND MONICA J. ROTH*

Department of Biochemistry, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Received 3 March 1994/Accepted 16 June 1994

Previous studies showed that an isolated human immunodeficiency virus type 1 (HIV-1) RNase H domain expressed as a fusion protein is highly active in Mn^{2+} , but activity was dependent on a hexahistidine tag located at either the carboxyl or amino terminus of the fusion protein (J. Smith and M. Roth, *J. Virol.* 67:4037-4049, 1993). It was postulated that a histidine tag can somehow provide a function normally associated with the DNA polymerase domain of HIV-1 reverse transcriptase. To determine the contributions of the DNA polymerase subdomains of HIV-1 reverse transcriptase to its RNase H activity, we have characterized the activity of isolated RNase H domains which include either portions of the connection, the entire connection, or both the thumb and connection as N-terminal extensions. Including increasing lengths of these domains at the N terminus of the RNase H resulted in a progressive increase in Mn^{2+} -dependent RNase H activity that was independent of a histidine tag. Activity of the isolated RNase H domains was also stimulated by the addition of independently purified polymerase subdomains. Further, this stimulation was shown to be a result of direct physical interactions between the thumb, connection, and RNase H domains. The connection and thumb subdomains were shown to contribute to substrate binding. The fingers and palm subdomains were found to be essential for Mg^{2+} -dependent RNase H activity.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, encodes a multifunctional reverse transcriptase (RT) which catalyzes the conversion of the viral single-stranded RNA genome into double-stranded DNA (for reviews, see references 7, 41, and 42). This enzyme is the target of several drugs, including the nucleoside analog 3'-azido-3'-deoxythymidine and the nonnucleoside compound Nevirapine, both of which act on the DNA polymerase activity (for a review, see reference 24). Less attention has been focused on the RNase H activity, which is responsible for degradation of the viral RNA as well as removal of the RNA primers for plus- and minus-strand DNA synthesis. The tRNA_{3'}^{Lys} minus-strand primer is specifically removed by the HIV-1 RT RNase H activity with an unusual specificity. Endonucleolytic cleavage occurs between the 3' terminal (A) and penultimate C residues of the tRNA, leaving the ribo A covalently linked to the U5 DNA terminus (13, 29, 34).

HIV-1 RT is an asymmetric heterodimer consisting of p51 and p66 subunits (10, 25). The p51 subunit and N-terminal 51 kDa of the p66 subunit are identical in primary sequence and form the DNA polymerase domain. The C terminus of the p66 subunit forms the RNase H domain. This domain is structurally very similar to the RNase H of *Escherichia coli*, even though the primary amino acid sequences are quite different (8, 20, 26, 43). The crystal structure of the intact heterodimer has revealed the existence of several subdomains in both the p51 and p66 subunits (1, 18, 21). These subdomains were related to the shape of a right hand and named, from N to C terminus, the fingers, palm, thumb, and connection (21).

Mutagenesis studies have determined that the DNA polymerase and RNase H activities are highly interdependent on each other (4, 15, 28). Indeed, according to the HIV-1 RT crystal structure, the RNase H domain has many contacts with the DNA polymerase domain (18, 21).

Unlike the isolated RNase H domain of Moloney murine leukemia virus and the RNase H of *E. coli*, most isolated RNase H domains of HIV-1 RT either are inactive or possess extremely low levels of activity (3, 17, 31, 37, 38). This lack of activity has been hypothesized to be due to the disorder of a loop containing a highly conserved histidine residue (H-539) and also the absence of substrate binding activity (8, 26, 27). There have also been reports of active RNase H domains (12, 14, 35). When the domain is expressed as a fusion protein with a polyhistidine tag at either the N or C terminus, RNase H activity can be detected when Mn^{2+} is supplied as the divalent cation (35). This RNase H domain (amino acids 427 to 560 or 400 to 560) produces the same specific endonucleolytic cleavage in removing the tRNA_{3'}^{Lys} minus-strand primer as the intact RT heterodimer, indicating that the protein determinants for specific tRNA_{3'}^{Lys} removal are intrinsic to the RNase H domain and independent of the DNA polymerase domain (35). Inactive RNase H produced without a histidine tag or with the histidine tag subsequently removed can have activity reconstituted by the addition of purified p51 subunit (12, 17, 35). It was therefore postulated that the histidine residues were providing a function normally provided by the DNA polymerase domain, either substrate binding or stabilization of the RNase H C terminus (35).

One of the major differences between HIV-1 RNase H and *E. coli* or Moloney murine leukemia virus RNase H is that the HIV-1 domain lacks a positively charged α helix C and adjacent loop which in *E. coli* has been shown to be important for substrate binding (19) and in Moloney murine leukemia virus RT has been shown to be important for DNA polymerase

* Corresponding author. Mailing address: Department of Biochemistry, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854. Phone: (908) 235-5048. Fax: (908) 235-4783. Electronic mail address: Roth@MBCL.Rutgers.Edu.

activity, suggesting it may be an interface between the RNase H and DNA polymerase (39) and important for cooperative DNA binding (40). Both the connection and thumb subdomains (C and T subdomains) of the DNA polymerase domain have major contacts with the RNase H domain and could potentially influence RNase H activity. Within the HIV-1 RT heterodimer, the region where α helix C is missing from the RNase H domain is replaced by the C subdomain (21). To determine the relative contributions of the C and T subdomains to RNase H activity, we have expressed and purified from *E. coli* the HIV-1 RNase H with N-terminal extensions from the DNA polymerase domain that include these subdomains. The addition of N-terminal sequences from the DNA polymerase domain resulted in a progressive increase in RNase H activity that did not require a histidine tag. The fingers-palm (FP), T, and C subdomains were purified as individual polypeptides and were found to stimulate activity when added in *trans* to the RNase H domain. The influences of the histidine tag and the DNA polymerase subdomains on the specific removal of the tRNA₃^{Lys} primer and substrate binding are examined.

MATERIALS AND METHODS

Materials. Purified HIV-1 RT p51 subunit was provided by Stuart Le Grice, Case Western Reserve University. [α -³⁵S]UTP was purchased from Amersham. [γ -³²P]ATP was purchased from ICN. Thrombin, glutathione-cross-linked agarose beads, and reduced glutathione were purchased from Sigma. Ni²⁺-nitrilotriacetate (NTA) resin was purchased from Qiagen (Chatsworth, Calif.).

Expression and purification of His₆ fusion proteins. To create the N-terminal extended RNase H expression plasmids, DNA fragments corresponding to the T and C subdomains of HIV-1 RT (HXB2 sequence) were PCR amplified with specific oligonucleotide primers. The N-terminal primers were complementary to either nucleotides 3869 to 3888 (NY441), 3707 to 3726 (Pro387), 3512 to 3533 (Ser322), or 3212 to 3230 (Gln222) and had an *Nde*I restriction site at the 5' end. The C-terminal primer was complementary to nucleotides 4058 to 4079, just 3' of an internal *Nsi*I site (nucleotide position 4050) within the RNase H domain. PCR amplification and subcloning into pTZ18u (U.S. Biochemical) was performed as described previously (35). An *Nde*I-*Nsi*I fragment was then subcloned into the *Nde*I-*Nsi*I sites of the RNase H expression vector pET-RHN2T (35), which has an N-terminal hexahistidine tag and a thrombin protease cleavage site. Individual FP, T, C, and thumb-connection (TC) subdomains were also PCR amplified from the RT DNA sequence and subcloned into the expression vector 6HisT-pET11 (16) as *Nde*I-*Bam*HI cassettes. Boundaries to yield stable subdomains were estimated from the crystal structure of HIV-1 RT from the laboratory of Edward Arnold (18).

Recombinant proteins except for the FP protein were purified from the *E. coli* expression strain BL21DE3 under denaturing conditions (4 M urea or 6 M guanidine-HCl) using Ni²⁺-chelate affinity chromatography and refolded as described previously (35). The T and FP polypeptides were purified under nondenaturing conditions. Expression conditions were the same, but the cells were sonicated in 30 mM Tris-HCl (pH 8.0)–50 mM KCl–10 mM β -mercaptoethanol–10% glycerol–1 mM phenylmethylsulfonyl fluoride–2 μ g of leupeptin per ml. Soluble material was bound to 0.5 ml of packed Ni²⁺-NTA resin for 1 h at 4°C on a rotating wheel and then washed with 10 ml of the same native buffer. Nonspecific binding proteins were stripped with two 10-ml washes of native

buffer with 1 M KCl followed by two 10-ml washes of the native buffer with 30 mM imidazole. The fusion proteins were eluted with 5 ml of native buffer supplemented with 150 mM imidazole. Protein fractions were dialyzed and stored in 50 mM Tris-HCl (pH 8.0)–50 mM KCl–2 mM dithiothreitol (DTT)–50% glycerol at –80°C. Following purification, the histidine tag was removed by thrombin cleavage when required (35).

Expression and purification of GST-fusion proteins. DNA fragments corresponding to the PCR products of the C, T, and TC subdomains were also ligated into the *Sma*I site of the glutathione S-transferase (GST) fusion vector pGEX-2T (Pharmacia), creating in-frame, C-terminal fusions with the coding sequence for GST (33). One-liter cultures of *E. coli* HB101 cells harboring these expression plasmids were induced with 0.1 mM isopropylthiogalactopyranoside (IPTG) for 2 h at 37°C. Cells were disrupted by sonication on ice in 15 ml of buffer S (30 mM Tris-HCl [pH 7.5], 150 mM KCl, 10% glycerol, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μ g of leupeptin per ml). Following centrifugation at 12,000 $\times$ g for 30 min at 4°C, 1.75 ml of a 50% slurry of glutathione-agarose beads was added, and the mixture was incubated for 1 h at 4°C. The beads were then washed four times with 5 ml of buffer S. Fusion proteins remained bound to the beads for binding experiments.

RNase H assays. Substrate for the quantitative, nonspecific RNase H assay was prepared by using circular single-stranded M13 phagemid DNA as a template for RNA synthesis by *E. coli* RNA polymerase (U.S. Biochemical), thus forming an RNA-DNA hybrid. Nonspecific RNase H assays were performed as previously described (35) except that the RNA portion of the substrate was labeled with [³⁵S]UTP instead of [³H]UTP and the standard reaction conditions consisted of 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 6.0 or 8.0), 4 or 8 mM MnCl₂, and 2 mM DTT. All reactions were stopped after the times indicated in each figure legend and fall within the linear range of activity to facilitate comparison of the various RNase H proteins. After trichloroacetic acid (TCA) precipitation and centrifugation, scintillation counting of acid-soluble radioactivity in the supernatant was used as a measure of RNase H activity. In reconstitution experiments, 4 pmol of either the RHN2T, Ser322, or Gln222 RNase H domain was preincubated with the various purified subdomains (4 pmol) for 5 min at 25°C prior to the addition of substrate to a final concentration of 2 to 3 nM. tRNA₃^{Lys} removal specificity assays were performed as previously described (34), using a model substrate consisting of the 18-mer 5'-end-labeled RNA oligonucleotide HPR-1 (5'-GUC CCUGUUCGGGCGCCA-3') annealed to the 38-mer DNA oligonucleotide HTD-1 (5'-GTGTGGAAAATCTCTAGCAG TGGCGCCCGAACAGGGAC-3') and extended with exonuclease-free Klenow polymerase (U.S. Biochemical). Reactions were performed at 37°C with the buffer conditions modified to 30 mM morpholineethanesulfonic acid (MES; pH 6.0)–12.5 mM KCl–8 mM MnCl₂–2 mM DTT.

Nucleic acid binding assays. Filter binding was performed as previously described (32), with the following modifications. Purified proteins were incubated with approximately 75,000 cpm (0.2 pmol) of ³²P-end-labeled RNA-DNA hybrid oligonucleotide substrates (HPR-1 and HTD-1) for 4 min on ice under the indicated buffer conditions in a volume of 20 μ l. Reaction mixtures were slowly passed through a 0.45- μ m-pore-size nitrocellulose filter (Millipore). Filters were then washed with 0.6 ml of the same binding buffer. Binding was quantitated by counting each filter on a liquid scintillation counter.

GST-RT fusion protein binding assays. The thumb (GST-T), connection (GST-C), and thumb-connection (GST-TC)

FIG. 1. Expression and purification of HIV-1 RT subdomains. (A) The subdomain organization of the p66 subunit of HIV-1 RT is shown as a schematic representation in which the F (fingers), P (palm), T (thumb), and C (connection) subdomains of the DNA polymerase domain are indicated. The RNase H domain of p66 makes up the C terminus. The polypeptides shown schematically were overexpressed in *E. coli* by using a T7 expression system. Each protein was tagged at the N terminus with six histidine residues adjacent to a thrombin protease cleavage site to facilitate removal of the histidines. Purification of the overexpressed protein was carried out on Ni^{2+} -NTA resin. The amino acid positions of the subdomain boundaries are shown at the top. The calculated molecular mass for each histidine-tagged polypeptide is also shown. The names of the amino-terminal extensions refer to the first RT-derived amino acid at the N terminus. NY427 and RHN2T (N-terminal amino acid Thr-400) have been purified previously (35). (B) SDS-polyacrylamide gel analysis of purified proteins. Approximately 5 μg of each purified protein was separated on a 15% gel and stained with Coomassie brilliant blue. Lane 1, NY441; lane 2, NY427; lane 3, RHN2T; lane 4, Pro387; lane 5, Ser322; lane 6, Gln222; lane 7, TC; lane 8, C; lanes 9 and 10, T; lane 11, FP. The proteins in lanes 1 to 9 were purified under denaturing conditions, and the proteins in lanes 10 and 11 were purified under nondenaturing conditions. Molecular weight markers are shown in lane M, with sizes in kilodaltons indicated at the left.

fusion proteins bound to glutathione-agarose beads (30 μl of a 50% slurry) were incubated with purified HIV-1 RNase H (4 μg) for 20 min at 4°C in a buffer consisting of 30 mM HEPES (pH 8.0), 50 mM KCl, and 2 mM DTT. Beads were then pelleted and washed five additional times with 50 μl of the same buffer. Proteins bound to the beads were stripped by the addition of 1% sodium dodecyl sulfate (SDS)-gel loading buffer containing 8 M urea and then resolved on SDS-15% polyacrylamide gel. Proteins remaining bound to the beads were detected by silver staining (Bio-Rad).

RESULTS

Protein purification. The predicted molecular mass for each of the polypeptides used in this study is shown in Fig. 1A. These RT polypeptides were purified under denaturing condi-

tions using Ni^{2+} -NTA columns as previously described for the isolated HIV-1 RNase H domain (35). In the cases of the T and FP polypeptides, nondenaturing conditions were also used. The peak fraction for each purified polypeptide is shown on a Coomassie blue-stained SDS-polyacrylamide gel in Fig. 1B. Each denatured fraction was refolded by step dialysis to remove the urea or guanidine-HCl and stored at -80°C . When necessary, the histidine tag was removed by proteolytic cleavage with thrombin (35).

The position of a histidine tag affects $\text{tRNA}_3^{\text{Lys}}$ removal specificity. Previous analysis of the isolated RNase H domain of HIV-1 RT demonstrated that it removes a model $\text{tRNA}_3^{\text{Lys}}$ minus-strand primer with the same unusual specificity as the wild-type p66/p51 heterodimer, wherein the 3' terminal ribo A of the tRNA remains linked to the U5 long terminal repeat terminus (34, 35). An oligonucleotide version of the substrate used in these assays is shown in Fig. 2A, in which the specific cleavage of the RNA (boldface) is indicated with an arrow. In Fig. 2B, the isolated RNase H domains RHN2T, RHC-1, NY427, and CY427, which have been purified and described previously (35), were assayed and compared for specific $\text{tRNA}_3^{\text{Lys}}$ removal. RHC-1 and CY427 are C-terminally histidine-tagged versions of RHN2T and NY427, respectively. The N-terminally tagged proteins produced the expected specificity in endonucleolytic cleavage (RHN2T, lanes 1 to 4; NY427, lanes 9 to 12). However, the C-terminally tagged proteins, RHC-1 and CY427, did not produce the same pattern of cleavage (lanes 5 to 8 and 13 to 16, respectively). Interestingly, the major product observed during cleavage with C-tagged RNase H is not the expected -1 17-mer product but rather is a band of 8 nucleotides which also accumulates during prolonged cleavage with the N-tagged RNase H. This product corresponds to cleavage in the middle of the RNA, between two U residues. While the major product observed with RHC-1 is the 8-mer, a smaller amount of the expected 17-mer product is also detected, which is different from that observed for CY427. The RNase H constructs initiating at amino acid 427 had approximately a 10-fold-greater specific activity than those initiating at amino acid 400. The reason for this difference in activity is currently unknown. These results suggest that a positively charged moiety positioned at the C terminus of RNase H disrupts proper positioning of the active site that is required for specific tRNA removal.

In Fig. 2C, the specificity of cleavage was tested on a substrate in which the HPR-1 model tRNA was not covalently linked to U5 DNA (lanes 5 to 8), thus altering a requirement for specific $\text{tRNA}_3^{\text{Lys}}$ removal (34). While the N-tagged RNase H, RHN-2, did not cleave this RNA-DNA hybrid (compare lane 7 with cleavage of the wild-type substrate in lane 2), the C-tagged RNase H, RHC-1, produced a nonspecific cleavage pattern similar to that observed with the wild-type substrate (compare lane 8 with cleavage of the model $\text{tRNA}_3^{\text{Lys}}$ removal substrate in lane 3). This altered specificity indicated that internal RNA cleavages produced by the C-tagged RNase H are independent of the structure of the U5/primer binding site junction. Although the N- and C-terminally tagged RNase H domains have the same activity level when acting on a nonspecific RNase H substrate (35), when presented with a specific substrate of defined sequence, the patterns of cleavage differ. These results also support the hypothesis that a polyhistidine tag can substitute for a region of the DNA polymerase N-terminal to the RNase H domain.

Histidine tag-independent RNase H activity. An RNase H consisting of amino acids 441 to 560 (NY441) corresponds to the domain which is produced by HIV-1 protease in the formation of p51 and does not contain any regions of the DNA

FIG. 2. Effect of histidine tag orientation on tRNA^{Lys} removal specificity. (A) Model substrate for determination of tRNA^{Lys} removal specificity. The substrate is a double-stranded 38-mer oligonucleotide which contains the HIV-1 primer binding site (PBS) adjacent to the U5 sequence. The first 3' 18 nucleotides of the minus-strand primer, tRNA^{Lys}, are hybridized to the plus-strand PBS and covalently linked to U5 sequences. The 5' end of the RNA within the minus strand is labeled with ³²P and indicated with an asterisk (34). RNA sequences are in boldface type. The expected cleavage site for correct tRNA^{Lys} removal by HIV-1 RNase H is indicated with an arrow. (B) Analysis of histidine tag positioning on cleavage specificity. Two picomoles of either N-terminal tagged RHN2T and NY427 or C-terminal tagged RHC-1 and CY427 were incubated with oligonucleotide substrate for the times indicated above each lane. Reactions were separated on a 20% sequencing gel. Lanes 1 to 4, RHN2T; lanes 5 to 8, RHC-1; lanes 9 to 12, NY427; lanes 13 to 16, CY427; lane M, full-length RNA (18 nucleotides long). The starting full-length substrate is 38 nucleotides long. The specific cleavage product is a 17-mer, and the secondary cleavage product is an 8-mer. (C) Nonspecific cleavages are independent of a covalent linkage between the tRNA and U5. tRNA cleavage by N-terminally tagged RHN-2 and C-terminally tagged RHC-1 on the wild-type substrate (lanes 1 to 4) was compared with the specificity of cleavage when the tRNA is not linked to DNA (lanes 5 to 8). The structure of the unlinked substrate is shown at the top. This substrate is similar to the wild-type substrate in panel A except that the minus-strand U5 DNA is missing. Lanes 1 and 5, no enzyme added (buffer only); lanes 2 and 7, incubated with RHN-2 (20.5 pmol); lanes 3 and 8, incubated with RHC-1 (17.5 pmol); lanes 4 and 6, incubated with equivalent volume of control fraction (pET11c expression vector). The starting substrate for lanes 1 to 4 was the 38-mer shown in panel A. The starting substrate for lanes 5 to 8 is an 18-mer. Sizes on panels B and C are indicated in nucleotides.

polymerase domain. This minimal RNase H domain had no detectable activity in our assays, even when histidine tagged (data not shown). To more clearly define the regions of the DNA polymerase domain that contribute to RNase H activity, activities of the N-terminally extended HIV-1 RNase H domains RHN2T, Pro387, Ser322, and Gln222 were compared after the histidine tag was removed by thrombin cleavage (Fig. 3). Pro387 extends 13 amino acids further N-terminal than

RHN2T. These 13 amino acids include the N terminus of α helix L and β strand 19 of the C subdomain. Ser322 includes all of the connection subdomain, and Gln222 includes all of the T subdomain and β strands 12, 13, and 14 of the palm. All three of these RT polypeptides have RNase H activity greater than the basal level detected for RHN2T in a nonspecific assay (Fig. 3A). A progressive increase in RNase H activity is gained as more of the DNA polymerase domain is included at the N

FIG. 3. Analysis of N-terminally extended RNase H domains for nonspecific and specific RNase H activity. (A) Protein titrations of nonspecific RNase H activity following thrombin cleavage to remove the histidine tags. [35 S]UTP-labeled RNA-DNA hybrid substrate was incubated with 0, 0.25, 0.5, 1.0, 2.0, and 4.0 pmol of either RHN2T, Pro387, Ser322, or Gln222 for 30 min at 37°C. Reactions were performed at pH 8.0. Activity is expressed as the percentage of the input substrate (0.15 pmol) that was cleaved into an acid-soluble form following TCA precipitation. (B) N-terminal extensions do not affect the specificity of tRNA removal. Two picomoles of Pro387 (lanes 1 to 4), Ser322 (lanes 5 to 8), and Gln222 (lanes 9 to 12) were incubated with the oligonucleotide tRNA removal substrate for the time indicated above each lane. Reaction conditions were the same as in Fig. 2B. Lane M, full-length 18-mer HPR-1 RNA. The primary cleavage product is a 17-mer, -1 species. Sizes are indicated in nucleotides.

terminus. This gain in activity is independent of the histidine tag, indicating that the region of the DNA polymerase domain for which the histidine tag substitutes is within the C and T subdomains. The C and T subdomains however, were not sufficient in restoring Mg^{2+} -dependent RNase H activity.

All three extended proteins without a histidine tag were able to specifically remove the tRNA₃^{Lys} primer as a 17-mer (Fig. 3B), indicating that the histidine tag is not directly responsible for the cleavage specificity observed in Fig. 2 for RHN2T and NY427 but that the C and T subdomains increase RNase H activity without disrupting the specificity of cleavage. If the N-terminal connection subdomain is replaced with GST as the fusion, nonspecific RNase H activity was retained but specific tRNA₃^{Lys} removal activity was greatly diminished (data not shown), indicating that nonspecific stabilization by an N-terminal GST fusion is not sufficient for tRNA₃^{Lys} removal.

Separately purified DNA polymerase subdomains stimulate the activity of RNase H domain. Given the overlap in primary amino acid sequence, the fingers and palm subdomains were purified as a single polypeptide (FP) that consisted of amino acids 1 to 221. The connection and thumb subdomains were purified as individual C and T polypeptides and also in tandem

as a single TC polypeptide (Fig. 1). To determine which regions of the DNA polymerase domain were responsible for the stimulation of Mn^{2+} -dependent RNase H activity that we observed previously in reconstitution reactions with RHN2T and p51 (35), the purified subdomains were preincubated with the HIV-1 RNase H domain, RHN2T, prior to the addition of substrate. As can be seen in Fig. 4A, Mn^{2+} -dependent RNase H activity was greatly stimulated by p51 as expected. While the T and FP subdomains had little effect on activity, the C subdomain stimulated activity. The TC tandem polypeptide reproducibly resulted in a slightly higher level of stimulation than the C polypeptide alone. In addition, this stimulation of activity was independent of the histidine tag on the subdomains (data not shown). Bovine serum albumin and GST did not affect RNase H activity (data not shown), suggesting that the stimulation was caused by specific interactions between the C, T, and RNase H domains.

The N-terminal extended RNase H (Ser322) has the C subdomain already present and presumably in the proper orientation relative to the RNase H domain. Using this protein as a base, the purified polymerase subdomains were mixed in the RNase H reaction (Fig. 4B). Again, p51 stimulated the Mn^{2+} -dependent RNase H activity, but because of the already high basal activity of Ser322, stimulation was not as drastic as in Fig. 4A. The TC polypeptide produced the best stimulation of the individual polypeptides, while the FP polypeptide had no effect on activity. However, a combination of the TC and FP subdomains added to Ser322 resulted in stimulation approximately equal to that observed with p51, indicating that the fingers and palm indirectly influence RNase H activity. The C subdomain within the Ser322 construct appears to play a role in positioning the other subdomains in the proper orientation.

Ser322 and Gln222 do not have significant levels of Mg^{2+} -dependent RNase H activity. It therefore seemed likely that the fingers and palm subdomains would be involved in this activity. Since neither the fingers nor palm subdomain contacts the RNase H domain within a heterodimer (18, 21), their influence on RNase H activity would be indirect, through interactions with the T and C subdomains to either form the primer/template binding cleft or influence the positioning of the thumb. As shown in Fig. 4C, low levels of Mg^{2+} -dependent RNase H activity can be restored to Ser322 and Gln222 by the addition of both TC and FP in the same reaction mixture but not by TC or FP individually. When p51 is added to Ser322, only a slight increase in stimulation is observed over that of FP plus TC. However, p51 produced a dramatic increase in the Mg^{2+} -dependent RNase H activity of Gln222, indicating that it is beneficial for the T subdomain to be in *cis* with the RNase H domain for Mg^{2+} -dependent cleavage.

Physical interactions between the RNase H and polymerase subdomains. The preceding experiments have provided evidence for the importance of contacts of the RNase H domain with not only the thumb but also the connection. According to the crystal structures of HIV-1 RT (18, 21), there should be important contacts between the RNase H, connection, and thumb. GST pulldown assays were used in identifying direct associations between the individual RT subdomains in solution. The T, C, and TC constructs were overexpressed and purified from *E. coli* as GST fusion proteins and are shown schematically in Fig. 5A. These fusion proteins bound to glutathione-agarose beads were added to RNase H reactions without added nucleic acid substrate and then pelleted to pull down interacting RNase H protein (Fig. 5B). Beads were added to either NY441 (amino acids 441 to 560) or RHC-1 (amino acids 400 to 560). RHC-1 includes most of the tryptophan-rich motif upstream of the RNase H domain and is

FIG. 4. Stimulation of isolated RNase H domain activity by purified DNA polymerase subdomains. RHN2T, Ser322, and Gln222 were pretreated with thrombin to remove the N-terminal histidine tag. (A) Reconstitution of Mn^{2+} -dependent RNase H activity to RHN2T. Four picomoles of RHN2T was preincubated with 4 pmol of the indicated protein for 5 min at 25°C in the absence of substrate and then incubated for 20 min at 37°C after the addition of [^{35}S]UTP-labeled nonspecific substrate. (B) Stimulation of the Mn^{2+} -dependent RNase H activity of Ser322. Four picomoles of Ser322 (S322) was preincubated with 4 pmol of the indicated protein(s) as in panel A. After the addition of substrate, 12.5- μ l aliquots were removed from the reaction and TCA precipitated at 2.5-min intervals for up to 10 min. (C) Reconstitution of Mg^{2+} -dependent RNase H activity to Ser322 and Gln222. Four picomoles of Ser322 or Gln222 was preincubated for 5 min with 4 pmol of the indicated protein(s). Reactions were carried out for 20 min after the addition of substrate, with Mg^{2+} as the divalent cation. Activity is expressed in each panel as the percentage of [^{35}S]UMP released from the starting substrate (0.15 pmol) as TCA-soluble counts.

tagged at the C terminus with six histidine residues (35). As shown in Fig. 5B, NY441 strongly interacted with both the C (lane 5) and TC subdomains (lane 6) but weakly with the T subdomain (lane 4). Very little NY441 bound nonspecifically to the glutathione beads without bound fusion protein or beads bound only to GST (lanes 2 and 3). Comparison of the bound and unbound RNase H revealed that the majority of the input NY441 was pulled down by the C and TC fusion proteins (data not shown). RHC-1 also bound to the T (lane 10), C (lane 11), and TC (lane 12) proteins in similar ratios as observed with NY441 but with an overall lower affinity. The RHN2T band was confirmed to be HIV-1 RNase H by Western blotting (immunoblotting) (data not shown). The reduction in binding affinity by RHC-1 suggests that the N-terminal extension of amino acids 400 to 440 may interfere to a certain extent with RNase H-connection interactions. A major secondary fusion protein was produced in the GST-C preparations. RNase H-connection interactions were insensitive to high salt conditions (150 mM KCl), but RNase H-thumb interactions were sensitive (data not shown). Along with the data in Fig. 4, these results indicate the importance of interactions between the

RNase H domain and regions of the DNA polymerase domain in RNase H activity.

The polymerase subdomains function in substrate binding. To test if the connection and thumb play a role in substrate binding, purified RT polypeptides were analyzed in a nitrocellulose filter binding assay (32). Prior to binding, the N-terminal polyhistidine tag was removed from each protein. As shown in Fig. 6, the isolated RNase H domain RHN2T did not bind efficiently to an oligonucleotide RNA-DNA substrate. In contrast, the N-terminally extended Ser322 and Gln222 constructs were shown to bind substrate. To determine if the C and T subdomains contributed directly to this observed binding, the isolated T, C, and TC polypeptides were also assayed for binding. The C and TC polypeptides bound this substrate at an intermediate level, indicating that the T and C subdomains do contribute to the binding of Ser322 and Gln222. The T subdomain did not have significant levels of binding. However, the TC polypeptide bound substrate slightly better than the C polypeptide, suggesting that the T subdomain may be dependent on the C subdomain to function properly. The presence of a histidine tag on the N terminus of each of the subdomains

FIG. 5. GST pulldown assay. The T, C, and TC subdomains were expressed in *E. coli* as carboxyl-terminal GST fusions and purified on glutathione-agarose beads. (A) Schematic representation of the purified GST fusion proteins. GST is shown as a white bar at the amino terminus. The T subdomain is shaded, and the C subdomain is striped. Calculated molecular sizes in kilodaltons are shown in parentheses. (B) RNase H interacts with the C and T subdomains. Four micrograms of purified NY441 or RHC-1 was incubated with an equal volume (50% slurry) of GST fusion beads (approximately 4 μ g of fusion protein) at 4°C for 20 min as described in Materials and Methods. Beads were pelleted and washed five times with 50 μ l of the binding buffer. Protein remaining bound to the beads was separated by electrophoresis on an SDS-15% polyacrylamide gel. Bound proteins were detected by silver staining of the gel. Lanes 1 and 7, 1- μ g markers (M) of NY441 and RHC-1, respectively; lanes 2 to 6, NY441 pulled down by agarose beads, GST, GST-T, GST-C, and GST-TC, respectively; lanes 8 to 12, RHC-1 pulled down by agarose beads, GST, GST-T, GST-C, and GST-TC, respectively. Fusion proteins are the uppermost bands in each lane.

described in Fig. 6 increased the overall binding affinity of each polypeptide, indicating that the histidine tag can provide a nonspecific binding function (data not shown). In addition, binding observed in these assays was salt sensitive (data not shown).

A schematic drawing of the HIV-1 RT heterodimer is shown in Fig. 7 and can be used as a guide to visualizing the potential interactions between the various combinations of subdomains described in this report. This representation emphasizes the extensive intersubdomain interactions that occur in the wild-type enzyme and also depicts the formation of the primer/template binding cleft by the fingers, palm, and thumb subdomains of the p66 subunit. Because of the complexity of the intersubunit interactions and the different structures of the two subunits within the heterodimer, the nature of the interactions between the subdomains used in this study is currently not known.

DISCUSSION

Previous analysis has led to the proposal that a histidine tag at the N terminus of an isolated HIV-1 RNase H domain can provide a function(s) normally associated with the DNA polymerase domain (35). In this study, we have investigated the effects of the various DNA polymerase subdomains on RNase H activity in terms of metal dependence and substrate

FIG. 6. Nucleic acid filter binding assay. Various concentrations of purified RHN2T, Ser322, Gln222, C, T, and TC proteins were analyzed for RNA-DNA hybrid binding at pH 6.0. Following removal of the N-terminal histidine tag, each protein was incubated for 4 min on ice with 0.2 pmol of 32 P-labeled oligonucleotide RNA-DNA hybrid in a 20- μ l reaction mixture containing 30 mM HEPES (pH 6.0), 5 mM KCl, and 2 mM DTT. Reaction mixtures were then slowly passed through a nitrocellulose filter and subsequently washed with 0.6 ml of the binding buffer. The percentage of the input substrate remaining bound to the filter was quantitated on a liquid scintillation counter. The oligonucleotide substrate used was the same as that shown at the top of Fig. 2C.

binding. We have also further analyzed the specificity of tRNA_{Lys} removal catalyzed by isolated HIV-1 RNase H domains and the contributions of the T and C subdomains to this specific cleavage activity.

The findings of this study have shown that contacts between the connection and RNase H domain are important for efficient RNase H activity (specific and nonspecific). The buried surface area between the connection and RNase H domain of the p66 subunit is 960 $\text{\AA}^2$ (96 nm). In terms of intersubdomain interactions, this is surpassed only by the surface area between the fingers and palm subdomains, which is 1,050 $\text{\AA}^2$ (105 nm) (11). This large buried surface area is reflected in the strong interactions observed between the RNase H domain and a GST-C fusion protein (Fig. 5) as well as the stimulation of Mn²⁺-dependent RNase H activity by the

FIG. 7. Schematic representation of the HIV-1 RT heterodimer and the intersubdomain associations. The p66 and p51 subunits are indicated at the left and are shaded differently. The RNase H domain of p66 is labeled R. The polymerase subdomains are labeled as follows: fingers (F), palm (P), thumb (T), and connection (C). Each purified subdomain has the potential to function in either a p66 or p51 orientation. For example, the thumb of p66 does not contact the RNase H domain, but the p51 thumb does contact the RNase H.

C subdomain added in *trans* (Fig. 4). In contrast, GST does not stimulate RNase H activity in *trans* or interact strongly with the RNase H domain. However, a GST-RNase H fusion protein was highly active in Mn^{2+} in a nonspecific assay but not in the tRNA removal assay (data not shown). Taken together, these data suggest that one role of the C subdomain is to stabilize the RNase H domain through their linkage in primary amino acid sequence. It is also possible that the added polymerase subdomains stimulate RNase H activity indirectly through providing a nucleic acid binding function. Filter binding data indicate that the connection can contribute to substrate binding (Fig. 6). This observation may explain why Ser322 (connection-RNase H) is highly active for tRNA removal using an oligonucleotide substrate but a GST-RNase H fusion is not. Filter binding studies were performed with a small oligonucleotide substrate. The X-ray crystal structure of HIV-1 RT cocrystallized with double-stranded DNA reveals potential contacts of the DNA with β strand 18 of the p66 connection subdomain (18). This β strand is positively charged, which could explain the salt sensitivity that we observed for substrate binding. The salt sensitivity of the connection subdomain agrees with previous observations that a polypeptide consisting of amino acids 303 to 560 and containing the C subdomain did not show nucleic acid binding in Northwestern (RNA-protein) or Southwestern (DNA-protein) blotting experiments at high salt conditions (23).

p51 thumb/p66 RNase H contacts (intermolecular) appear to stabilize a C-terminal loop of the RNase H domain, which contains a conserved histidine residue (H-539) (21). However, an isolated T subdomain did not have any effect on RNase H activity (Fig. 4) and weakly interacted with the RNase H domain when fused with GST (Fig. 5). The isolated T subdomain may be structurally unstable and rely on contacts with other subdomains. This hypothesis is supported by the finding that the TC construct slightly increases not only Mn^{2+} -dependent RNase H activity but also substrate binding. Further, the thumb present on Gln222 allowed for greater recovery of Mg^{2+} -dependent RNase H activity, by reconstitution with p51 (Fig. 4C).

The RNase H activity of HIV-1 RT heterodimer prefers Mg^{2+} over Mn^{2+} as a divalent cation. The reason for this preference is not known. However, Mn^{2+} can also be utilized. Within the crystal structure of an isolated RNase H domain, two Mn^{2+} cations are bound at the active site (8). The isolated RNase H domains analyzed in this study were active only in the presence of Mn^{2+} . Restoration of Mg^{2+} -dependent RNase H activity to Ser322 or Gln222 required the presence of both the FP and TC subdomains. The requirement for both of these polypeptides or p51 suggests that the FP and TC subdomains may interact together to re-form a p51 subunit. The fingers and palm subdomains also comprise a large portion of the DNA polymerase active site and the nucleic acid binding cleft within the p66 subunit. It is therefore equally possible that the combination of FP and TC subdomains could enhance substrate binding. Point mutations in the fingers subdomain that do not affect DNA polymerization but eliminate RNase H activity have been proposed to alter the positioning of the primer/template (5).

The RNase H domain of HIV-1 RT has previously been shown to bind poly(rA)-oligo(dT) with an apparent binding constant in the micromolar range. The intrinsic binding constant was calculated at only $3.9 \times 10^3 M^{-1}$ due to a large cooperativity contribution (6). However, the K_d for HIV-1 RT heterodimer has been calculated by several groups to be in the low nanomolar range (9, 22, 30). The inactivity of the isolated HIV-1 RNase H domain has been proposed to be at least

partially due to a lack of substrate binding (8). From the results of this study, the cleavage specificity of HIV-1 RNase H does not appear to be affected by the mode of substrate binding when the orientation of substrate binding is maintained properly. UV cross-linking of oligo(dT)₁₆ or oligo(dT)₁₅ to HIV-1 RT has implicated the region between amino acids 195 and 300 within the T subdomain as being in close proximity to a substrate binding pocket (2, 36). Indeed, the thumb of p66 contacts the sugar-phosphate backbone of both the template and primer strands (18). Contacts between the p66 subunit and double-stranded DNA within the heterodimer are distributed throughout the subdomains in terms of the buried surface area between DNA and protein (11), indicating that all of the subdomains contribute to primer/template binding.

In summary, we have demonstrated that the connection and thumb subdomains are important for Mn^{2+} -dependent RNase H activity of HIV-1 RT. The connection was shown to stimulate Mn^{2+} -dependent activity both in *cis* and in *trans* as well as to interact directly with the RNase H domain. The presence of the T subdomain slightly enhanced the effects of the C subdomain. These subdomains increased overall RNase H activity without affecting the specificity of tRNA_{3^{lys}} removal. Further analyses of the protein and nucleic acid determinants for tRNA_{3^{lys}} removal are ongoing.

ACKNOWLEDGMENTS

This work was supported by grant R01-CA49932 from the National Institutes of Health and grant 02124-15-RG from AmFAR. M.J.R. is a recipient of a Leukemia Society of America, Inc., Scholar Award. J.S.S. was supported by New Jersey Commission on Cancer Research predoctoral fellowship 691-037-F2. K.G. was supported by the Rutgers College Henry Rutgers Scholars Program.

We thank Edward Arnold and Alfredo Jacobo-Molina for structural information of HIV-1 RT complexed with double-stranded DNA. We thank Paul March and Millie Georgiadis for critical reading of the manuscript.

REFERENCES

1. Arnold, E., A. Jacobo-Molina, R. G. Nanni, R. L. Williams, X. Lu, J. Ding, J. Arthur D. Clark, A. Zhang, A. L. Ferris, P. Clark, A. Hizi, and S. H. Hughes. 1992. Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. *Nature (London)* **357**:85–89.
2. Basu, A., K. K. Ahluwalia, S. Basu, and M. J. Modak. 1992. Identification of the primer binding domain in human immunodeficiency virus reverse transcriptase. *Biochemistry* **31**:616–623.
3. Becerra, S. P., G. M. Clore, A. M. Gronenborn, A. R. Karlstrom, S. J. Stahl, S. H. Wilson, and P. T. Wingfield. 1990. Purification and characterization of the RNase H domain of HIV-1 reverse transcriptase expressed in recombinant *Escherichia coli*. *FEBS Lett.* **270**:76–80.
4. Boyer, P. L., A. L. Ferris, and S. H. Hughes. 1992. Cassette mutagenesis of the reverse transcriptase of human immunodeficiency virus type 1. *J. Virol.* **66**:1031–1039.
5. Boyer, P. L., A. L. Ferris, and S. H. Hughes. 1992. Mutational analysis of the fingers domain of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **66**:7533–7537.
6. Cirino, N. M., R. C. Kalayjian, J. E. Jentoft, and S. F. J. Le Grice. 1993. Fluorimetric analysis of recombinant p15 HIV-1 ribonuclease H. *J. Biol. Chem.* **268**:14743–14749.
7. Coffin, J. M. 1990. Retroviridae and their replication, p. 1437–1500. In B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), *Virology*, 2nd ed. Raven Press, Ltd., New York.
8. Davies, J. F., II, Z. Hostomska, Z. Hostomsky, S. R. Jordan, and D. A. Matthews. 1991. Crystal structure of the ribonuclease H

- domain of HIV-1 reverse transcriptase. *Science* **252**:88–95.
9. DeStefano, J. J., R. A. Bambara, and P. J. Fay. 1993. Parameters that influence the binding of human immunodeficiency virus reverse transcriptase to nucleic acid structures. *Biochemistry* **32**:6908–6915.
 10. DiMarzo Veronese, F., T. D. Copeland, A. L. DeVico, R. Rahman, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan. 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**:1289–1291.
 11. Ding, J., A. Jacobo-Molina, C. Tantillo, X. Lu, R. G. Nanni, and E. Arnold. Buried surface analysis of HIV-1 reverse transcriptase p66/p51 heterodimer and its interaction with dsDNA template/primer. *J. Mol. Recognition*, in press.
 12. Evans, D. B., K. Brawn, J. Martin, R. Deibel, W. G. Tarpley, and S. K. Sharma. 1991. A recombinant ribonuclease H domain of HIV-1 reverse transcriptase that is enzymatically active. *J. Biol. Chem.* **266**:20583–20585.
 13. Furfine, E. S., and J. E. Reardon. 1991. Human immunodeficiency virus reverse transcriptase ribonuclease H: specificity of tRNA^{Lys3}-primer excision. *Biochemistry* **30**:7041–7046.
 14. Hansen, J., T. Schulze, W. Mellert, and K. Moelling. 1988. Identification and characterization of HIV-specific RNase H by monoclonal antibody. *EMBO J.* **7**:239–243.
 15. Hizi, A., S. H. Hughes, and M. Shaharabany. 1990. Mutational analysis of the ribonuclease H activity of human immunodeficiency virus 1 reverse transcriptase. *Virology* **175**:575–580.
 16. Hoffman, A., and R. G. Roeder. 1991. Purification of his-tagged proteins in non-denaturing conditions suggests convenient method for protein interaction studies. *Nucleic Acids Res.* **19**:6337–6338.
 17. Hostomsky, Z., Z. Hostomska, G. O. Hudson, E. W. Moomaw, and B. R. Nodes. 1991. Reconstitution *in vitro* of RNase H activity by using purified N-terminal and C-terminal domains of human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **88**:1148–1152.
 18. Jacobo-Molina, A., J. Ding, R. D. Nanni, J. Arthur, D. Clark, X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**:6320–6324.
 19. Kanaya, S., C. Katsuda-Nakai, and M. Ikehara. 1991. Importance of the positive charge cluster in *Escherichia coli* ribonuclease HI for the effective binding of the substrate. *J. Biol. Chem.* **266**:11621–11627.
 20. Katayanagi, K., M. Miyagawa, M. Matsushima, M. Ishikawa, S. Kanaya, M. Ikehara, T. Matsuzaki, and K. Morikawa. 1990. Three-dimensional structure of ribonuclease H from *E. coli*. *Nature (London)* **347**:306–309.
 21. Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783–1790.
 22. Krug, M. S., and S. L. Berger. 1991. Reverse transcriptase from human immunodeficiency virus: a single template-primer binding site serves two physically separable catalytic functions. *Biochemistry* **30**:10614–10623.
 23. Kumar, A., H. Kim, R. W. Sobol, S. P. Becerra, B. Lee, D. L. Hatfield, R. J. Suhadolnik, and S. H. Wilson. 1993. Mapping of nucleic acid binding in proteolytic domains of HIV-1 reverse transcriptase. *Biochemistry* **32**:7466–7474.
 24. Larder, B. A. 1993. Inhibitors of HIV reverse transcriptase as antiviral agents and drug resistance, p. 205–222. *In* A. M. Skalka, and S. P. Goff (ed.), *Reverse transcriptase*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 25. Lightfoot, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan. 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of acquired immunodeficiency syndrome virus. *J. Virol.* **60**:771–775.
 26. Powers, R., G. M. Clore, A. Bax, D. S. Garrett, S. J. Stahl, P. T. Wingfield, and A. M. Gronenborn. 1991. Secondary structure of the ribonuclease H domain of the human immunodeficiency virus reverse transcriptase in solution using three-dimensional double and triple resonance heteronuclear magnetic resonance spectroscopy. *J. Mol. Biol.* **221**:1081–1090.
 27. Powers, R., G. M. Clore, S. J. Stahl, P. T. Wingfield, and A. Gronenborn. 1992. Analysis of the backbone dynamics of the ribonuclease H domain of the human immunodeficiency virus reverse transcriptase using ¹⁵N relaxation measurements. *Biochemistry* **31**:9150–9157.
 28. Prasad, V. R., and S. P. Goff. 1989. Linker insertion mutagenesis of the human immunodeficiency virus reverse transcriptase expressed in bacteria: Definition of the minimal polymerase domain. *Proc. Natl. Acad. Sci. USA* **86**:3104–3108.
 29. Pullen, K. A., L. K. Ishimoto, and J. J. Champoux. 1992. Incomplete removal of the RNA primer for minus-strand DNA synthesis by human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **66**:367–373.
 30. Reardon, J. E., E. S. Furfine, and N. Cheng. 1991. Human immunodeficiency virus reverse transcriptase: effect of primer length on template-primer binding. *J. Biol. Chem.* **266**:14128–14134.
 31. Restle, T., B. Muller, and R. S. Goody. 1992. RNase H activity of HIV reverse transcriptase is confined exclusively to the dimeric forms. *FEBS Lett.* **300**:97–100.
 32. Roth, M. J., N. Tanese, and S. P. Goff. 1988. Gene product of Moloney murine leukemia virus required for proviral integration is a DNA-binding protein. *J. Mol. Biol.* **203**:131–139.
 33. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**:31–40.
 34. Smith, J. S., and M. J. Roth. 1992. Specificity of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H in removal of the minus-strand primer, tRNA^{Lys3}. *J. Biol. Chem.* **267**:15071–15079.
 35. Smith, J. S., and M. J. Roth. 1993. Purification and characterization of an active human immunodeficiency virus type 1 RNase H domain. *J. Virol.* **67**:4037–4049.
 36. Sobol, R. W., R. J. Suhadolnik, A. Kumar, B. J. Lee, D. L. Hatfield, and S. H. Wilson. 1991. Localization of a polynucleotide binding region in the HIV-1 reverse transcriptase: Implications for primer binding. *Biochemistry* **30**:10623–10631.
 37. Stammers, D. K., M. Tisdale, S. Court, V. Parmar, C. Bradley, and C. K. Ross. 1991. Rapid purification and characterisation of HIV-1 reverse transcriptase and RNase H engineered to incorporate a C-terminal tripeptide α -tubulin epitope. *FEBS Lett.* **283**:298–302.
 38. Tanese, N., and S. P. Goff. 1988. Domain structure of Moloney murine leukemia virus reverse transcriptase: mutational analysis and separate expression of the DNA polymerase and RNase H activities. *Proc. Natl. Acad. Sci. USA* **85**:1777–1781.
 39. Telesnitsky, A., S. W. Blain, and S. P. Goff. 1992. Defects in Moloney murine leukemia virus replication caused by a reverse transcriptase mutation modeled on the structure of *Escherichia coli* RNase H. *J. Virol.* **66**:615–622.
 40. Telesnitsky, A., and S. P. Goff. 1993. RNase H domain mutations affect the interaction between Moloney murine leukemia virus reverse transcriptase and its primer-template. *Proc. Natl. Acad. Sci. USA* **90**:1276–1280.
 41. Varmus, H. E., and R. Swanstrom. 1982. Replication of retroviruses, p. 75–134. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 42. Whitcomb, J. M., and S. H. Hughes. 1992. Retroviral reverse transcription and integration: progress and problems. *Annu. Rev. Cell Biol.* **8**:275–306.
 43. Yang, W., W. A. Hendrickson, R. J. Crouch, and Y. Satow. 1990. Structure of ribonuclease H phased at 2 Å resolution by MAD analysis of the selenomethionyl protein. *Science* **249**:1398–1405.