Analysis of the Ribonuclease H Activity of HIV-1 Reverse Transcriptase Using RNA·DNA Hybrid Substrates Derived from the *gag* Region of HIV-1†

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Received April 13, 1989; Revised Manuscript Received July 3, 1989

ABSTRACT: The RNase H activity associated with recombinant p66/p51 HIV-1 reverse transcriptase (RT) has been analyzed in the absence of DNA synthesis by using homogeneous RNA·DNA substrates. The substrates consisted of SP6 runoff transcripts from a portion of the *gag* region of the HIV-1 genome hybridized to complementary single-stranded DNA from either an M13 subclone or a phagemid transcription vector subclone. The corresponding hybrids either carried a 5′-mismatch of seven nucleotides or were fully base-paired. Analysis of recombinant HIV-1 p66/p51 RT by an activated gel assay employing these substrates suggested that the RNase H activity was exclusively associated with the p66 polypeptide. Denaturing gel electrophoresis was used to analyze the oligonucleotide products generated by hydrolysis of the hybrids by HIV-1 RT, M-MuLV RT, and *Escherichia coli* RNase H. The significant difference in the time-dependent distribution of products of HIV-1 RT vs *E. coli* RNase H catalyzed cleavage of 5′-mismatched hybrids indicated that the preparation of recombinant HIV-1 RT was free of contaminating bacterial RNase H. Although the HIV-1 RT associated RNase H activity shares many of the general mechanistic features of other retroviral enzymes [Gerard, G. F. (1981) *Biochemistry 20*, 256–265], the appearance of unique intermediates and end products in the course of hydrolysis of 5′-mismatched and fully base-paired hybrids indicated a significant difference in the sequence dependence of the kinetics of RNase H cleavage by HIV-1 RT and M-MuLV RT.

Human immunodeficiency virus 1 (HIV-1)[1] is the causative agent of AIDS (Gallo et al., 1983; Montagnier et al., 1984; Popovic et al., 1984). As with other retroviruses, the viral *pol* gene encoded reverse transcriptase is the enzyme that is solely responsible for replication of the viral RNA to produce a ds-DNA that can be integrated into the host genome (Hershey & Taylor, 1987). The three RT-associated activities that catalyze the necessary series of reactions are as follows: (i) tRNA-primed RNA-dependent (−)-strand DNA synthesis, (ii) ribonuclease H degradation of the intermediate RNA·DNA hybrid, and (iii) DNA-dependent (+)-strand DNA synthesis, primed at a position 5′ to the U3 region of the viral genome by a polypurine-rich oligonucleotide that is a RNase H digestion product of the viral RNA (Collett et al., 1978; Gilboa et al., 1979; Smith et al., 1984a,b; Resnick et al., 1984; Champoux et al., 1984). The RNase H activity is also responsible for specific removal of the tRNA and (+)-strand primers by specific cleavage at the RNA·DNA junctions of intermediates along the replication pathway (Omer & Faras, 1982; Champoux et al., 1984).

Analysis of the structure–function relationship between the polymerase and RNase H activities of M-MuLV RT has shown that the activities reside in distinct and separate portions of the molecule (Tanese & Goff, 1988). In this modular domain arrangement of M-MuLV RT, the RNase H domain is located at the COOH terminus (Kotewicz et al., 1988). Recent studies on viral and recombinant HIV-1 RT support a similar domain organization in which the polymerase domain is contained within the NH_2-terminal 51 kDa and the RNase H is located in the COOH-terminal 15 kDa of the p66 subunit of the p66/p51 heterodimer (Lowe et al., 1988; Tisdale et al., 1988; Hansen et al., 1988; Mizrahi et al., 1989).

The RT, protease, and endonuclease retroviral enzymes encoded by the *pol* gene are all obvious targets in the search for therapeutic agents against the spread of viral infection. Indeed, the widely prescribed drug 3′-azido-3′-deoxythymidine (AZT) is a potent inhibitor of the reverse transcriptase activity following in vivo conversion to the corresponding triphosphate (Mitsuya et al., 1985). To date, the RNase H activity has received less attention as a potential target for drug design and discovery despite its crucial role in the viral replication cycle (Tanese & Goff, 1988; Repaske et al., 1989). In this paper, the design and synthesis of RNA·DNA hybrid substrates of defined length and sequence for use in mechanistic and inhibitor screening studies is described, and their application in establishing the basic features of the RNase H activity of HIV-1 RT is discussed.

Materials and Methods

Materials

SP6 RNA polymerase, *Escherichia coli* RNase H (0.05 mg/mL; 38 000 units/mg), RNase inhibitor, and the tran-

† This research was funded in part by NIH Grant GM39526.

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[1] Abbreviations: HIV-1, human immunodeficiency virus type I; AIDS, acquired immune deficiency syndrome; M-MuLV, Moloney murine leukemia virus; AMV, avian myeloblastosis virus; RT, reverse transcriptase; RNase H, ribonuclease H; PPT, polypurine tract; DNase, deoxyribonuclease; bp, base pair; ds, double stranded; ss, single stranded; U3, unique 3′-terminal region; nt, nucleotide; RNAP, RNA polymerase; kDa, kilodalton(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AZT, 3′-azido-3′-deoxythymidine; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GSH, glutathione; MCS, multiple cloning site.

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scription vectors pGEM-3Zf(+) and pGEM-3Zf(−) were from Promega, RNase-free DNase I was from Boehringer-Mannheim, M-MuLV RT (0.24 mg/mL) was from Pharmacia, and T4 polynucleotide kinase and restriction enzymes were from Pharmacia and Boehringer-Mannheim. Authentic recombinant p66/p51 HIV-RT expressed in *E. coli* was kindly provided by G. Lazarus (0.45 mg/mL, >95% pure; Mizrahi et al., 1989). Molar concentrations of RNase H enzyme associated with HIV-1 RT were calculated from protein concentrations assuming one RNase H active site per 117-kDa p66/p51 heterodimer. [^{3}H]UTP (40–45 Ci/mmol) was from Amersham, and [^{3}H]dTTP (47 Ci/mmol), [α-^{32}P]UTP (600 Ci/mmol), [γ-^{32}P]GTP (1500 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from ICN. dTTP, ATP, CTP, GTP, and UTP were from Sigma, and $(dT)_{12-18}$ was from Pharmacia. Diethyl pyrocarbonate was from Sigma. Sephadex G-50 (DNA quality, medium) was from Pharmacia, DE81 filter disks were from Whatman, and liquid scintillation cocktail was from Du Pont. All other reagents were the highest grade commercially available.

Plasmids and Bacterial Strains. *E. coli* DH5α [a derivative of DH-1 (Hanahan, 1983)] and JM101 or JM109 (Yanisch-Perron et al., 1985) were used for pGEM-3Zf(+)/pGEM-3Zf(−) and M13mp19 cloning, respectively. DNA manipulations were carried out according to standard protocols (Maniatis et al., 1982). Clones were sequenced by the dideoxy method (Sanger et al., 1977). The 627-bp *Hin*dIII *gag* fragment [positions 629–1256; numbering according to Ratner et al. (1985)], kindly provided by C. Debouck, was from the BH10 clone of the HTLVIIIB isolate of HIV (Shaw et al., 1984). The fragment was cloned in both orientations into the *Hin*dIII sites of pGEM-3Zf(+), pGEM-3Zf(−), and M13mp19 to yield the clockwise (C) and anticlockwise (A) pairs of clones pGEMf(+)-GAG-C/pGEMf(+)-GAG-A, pGEMf(−)-GAG-C/pGEMf(−)-GAG-A, and M13-GAG-C/M13-GAG-A, respectively.

Methods

RNase H Substrate Preparation. (*1*) *SP6 Transcription.* For the preparation of uniformly labeled transcripts, reactions (40 μL) containing 40 mM Tris·HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 1 unit/μL RNase inhibitor, 750 μM each ATP, CTP, and GTP, 20–50 μM [α-^{32}P]UTP (60 Ci/mmol) or 50 μM [^{3}H]UTP (40–45 Ci/mmol), linear DNA template (0.2 μg/μL), and SP6 RNAP (2 units/μL) were incubated at 40 °C for 2.5 h. For the preparation of 5′-end-labeled transcripts, the nucleotide mixture consisted of 750 μM each ATP, CTP, and UTP and 20 μM [γ-^{32}P]GTP. DNase I was added to a final concentration of 0.7 unit/μL, and the mixture was incubated at 37 °C for 15 min and then diluted with 100 μL of TE buffer [10 mM Tris·HCl (pH 8.0)/0.1 mM EDTA]. The mixture was extracted with phenol/$CHCl_3$ (1/1) and then loaded onto two identical 0.85-mL Sephadex G-50 syringe spin columns (70 μL per column; equilibrated in TE), which were centrifuged in a swinging bucket rotor for 3 min, resulting in >99.9% removal of unincorporated mononucleotides. A control experiment using a ^{32}P-labeled DNA template demonstrated that the spin column procedure reproducibly removed 90% of the DNase I digestion products. The overall recovery of label as RNA transcript following purification was 35–50% (ca. 3×10^5 dpm/μL product). The full-length ^{32}P-labeled transcripts were analyzed on 6% polyacrylamide/8 M urea gels prior to further use and were found to be at least 85% homogeneous. Although the full-length RNA was contaminated with several small oligonucleotides (5–10 nt) which were possibly premature transcription termination products, no attempt was made to further purify the RNA prior to hybridization (see, for example, Figure 2, lanes 1, 7, and 13; Figure 3, lanes 1 and 6; and Figure 4, lanes 2 and 8).

(*2*) *ss-DNA Isolation.* ss-M13 DNA was isolated from phage-containing supernatants as previously described (Kunkel et al., 1987). Single-stranded plasmid DNA fully complementary to the (+)-GAG^{71-691} transcripts was isolated from the supernatant of a culture of JM109/pGEMf(−)-GAG-A superinfected with the helper phage M13KO7 (Vieira & Messing, 1987).

(*3*) *Hybridization.* Mixtures containing labeled transcript, a 2–10-fold molar excess of complementary ss-DNA, 40 mM Tris·HCl (pH 7.9), 40 mM KCl, and 4 mM $MgCl_2$ were placed in a water bath initially at 55–60 °C, allowed to cool slowly to room temperature (1–2 h), and stored at −20 °C. Hybrid formation was monitored by analyzing aliquots on a 6% nondenaturing polyacrylamide gel. Since the yield of ss-DNA from the phagemid pGEMf(−)-GAG-A was 5–10-fold lower than from the phage M13-GAG-A, the latter was used for large-scale production of the hybrids described in this work. As a result, all hybrids between (+)-GAG^{71-691} and ss-M13-GAG-A carried a 7-nt 5′-mismatch corresponding to the region between the SP6 transcriptional initiation site within the pGEM-GAG-A vector and the *Hin*dIII site of insertion of the *gag* fragment (Figures 1 and 5). Moreover, the DE81 filter assay substrate (+)-GAG^{691}/ss-M13-GAG-A also carried a 3′-terminal mismatch of 51 nt corresponding to the noncomplementary *Hin*dIII–*Eco*RI region of the MCS of the pGEM-GAG-A vector (Figure 1). Fully complementary hybrids were prepared by hybridization of (+)-GAG^{71-691} to ss-DNA isolated from the phagemid subclone pGEMf(−)-GAG-A. In this subclone, the orientation of the phage f1 origin of replication within the vector was clockwise, and as such, superinfection with the helper phage M13K07 resulted in the production of a single-stranded copy of the pGEM-GAG-A plasmid of which a portion was complementary to the entire length of the (+)-GAG^{71-691} transcripts, including the 8 nt between the SP6 RNAP transcriptional initiation site and the start of the *gag* sequence (Figure 1).[2]

RNase H Assays. (*1*) *Filter Assay.* Unless otherwise indicated, reaction solutions (100 μL) containing 50 mM Tris·HCl (pH 7.9), 50 mM KCl, 7 mM $MgCl_2$, 5 mM DTT, 2 mM GSH, ^{3}H-labeled hybrid (ca. 20000 dpm/μL, prepared as described above), and either 2–100 nM HIV-1 RT or 0.05–0.5 nM *E. coli* RNase H were incubated at 37 °C and quenched with 20 μL of 0.1 M EDTA after 1–60 min. Assay solutions for RNase H reactions catalyzed by M-MuLV RT contained 50 mM Tris·HCl (pH 7.9), 50 mM KCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 5 mM DTT, ^{3}H-labeled hybrid (20000 dpm/μL), and 0.5–40 nM M-MuLV RT. Quenched aliquots were applied to 2.5-cm DE81 filter disks, washed twice with 0.5 M Na_2HPO_4 and once with 0.3 M ammonium formate (pH 8.0), followed by ethanol and ether (10 mL/filter), and then air-dried, and their radioactivity was measured by liquid scintillation counting (Beckman LS 5801) in 5 mL of scintillation cocktail.

(*2*) *Activated Gel Assay.* SDS gels (Laemmli, 1970) were copolymerized with ^{32}P-labeled hybrid (ca. 4×10^6 dpm) in

[2] Although the distance between the transcriptional initiation site and the *Hin*dIII site of insertion of the *gag* fragment was 8 bp, the actual 5′-mismatch in the hybrids (+)-GAG^{71-691}/ss-M13-GAG-A was only 7 nt as a result of fortuitous complementarity between the eighth base (C) in the 71–691-nt RNA transcripts and the corresponding residue in the M13-GAG-A sequence (G).

a 10% polyacrylamide resolving gel. Following electrophoresis, gels were soaked without agitation for 4–5 days at 22 °C in five changes of assay buffer [50 mM Tris·HCl (pH 7.9)/50 mM NaCl/8 mM $MgCl_2$/2 mM DTT/3% glycerol] and then dried and exposed to X-ray film. Under these conditions, sharply defined clearance zones were observed for both HIV-1 RT (p66 position) and *E. coli* RNase H. In contrast, significantly reduced sensitivity and definition were observed for HIV-1 RT at higher assay temperatures (33–37 °C).

(3) Gel Electrophoretic Assay. Reactions (40 μL) containing 50 mM Tris·HCl (pH 7.9), 50 mM KCl, 7 mM $MgCl_2$, 5 mM DTT, 2 mM GSH, ^{32}P-labeled hybrid (ca. 10^6 dpm), and either HIV-1 RT or *E. coli* RNase H were incubated at 37 °C. Reactions catalyzed by M-MuLV RT were conducted under the same conditions as described for the filter assay, except ^{32}P-labeled hybrid was used instead of the ^3H-labeled substrate. Enzyme preparations were diluted with storage buffer [50 mM Tris·HCl (pH 8.0)/0.1 M NaCl/5 mM DTT/1 mM EDTA/0.1% Triton X-100/50% glycerol] 2–5 min prior to initiating the reactions. Aliquots (6.4 μL) were withdrawn after 0.5–60 min and quenched with 13 μL of sample loading buffer [80% deionized $HCONH_2$/0.1% bromophenol blue/0.1% xylene cyanol/TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA)]. Quenched samples were heated at 95 °C for 3–5 min, and aliquots (2 μL) were loaded onto a 30 cm × 40 cm × 0.4 mm 15% polyacrylamide gel containing 8 M urea in TBE buffer. The size markers $p(dT)_{12-18}$ were prepared by 5′-end labeling $(dT)_{12-18}$ with T4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). Following electrophoresis (2.5 h, 1800 V), the gels were lifted onto film, covered with Saran wrap, and autoradiographed with an intensifier screen at −70 °C.

Results

Hybrid Design and Preparation. The enzymatically synthesized SP6 runoff transcripts corresponding to portions of (+)-strand and (−)-strand HIV-1 *gag* RNA are shown in Figure 1. Hybridization with ss-DNA from either a complementary M13 subclone or a pGEM transcription vector subclone was monitored by nondenaturing gel electrophoresis, which indicated that complete hybrid formation between (+)-GAG^{691} and complementary ss-M13-GAG-A required a molar excess of DNA over RNA of at least 3-fold (data not shown). Ribonucleolytic degradation of the resulting hybrid was followed by a DE81 filter assay. The HIV-1 RT catalyzed cleavage of the (+)-GAG hybrid removed up to 90% of the label at a rate dependent upon the enzyme concentration (data not shown). The rate was independent of the concentration of ss-DNA used in the hybrid preparation (2–10-fold molar excess over [RNA]), suggesting negligible nonspecific binding of the enzyme to ss-DNA. Furthermore, the lack of digestion of a negative control substrate prepared from the (−)-GAG^{578} RNA and homologous ss-M13-GAG-A confirmed that the observed loss of label was dependent on RNA·DNA hybrid formation (data not shown).

Activated Gel Assay. The hybrid substrates were used to develop an activated gel RNase H assay, which was based on previously described procedures (Carl et al., 1980; Crouch & Dirksen, 1985; Tanese & Goff, 1988). The assay was used to visualize the activities of *E. coli* RNase H and recombinant HIV-1 RT (Mizrahi et al., 1989). Although the HIV-1 RT preparation consisted of an equimolar mixture of p66 and p51 polypeptides that share a common NH_2 terminus (Mizrahi et al., 1989), the RNase H activity was apparently only associated with p66, since no clearance was seen at the p51 position, even in overloaded lanes (>100 μg of HIV-1 RT; data not shown).

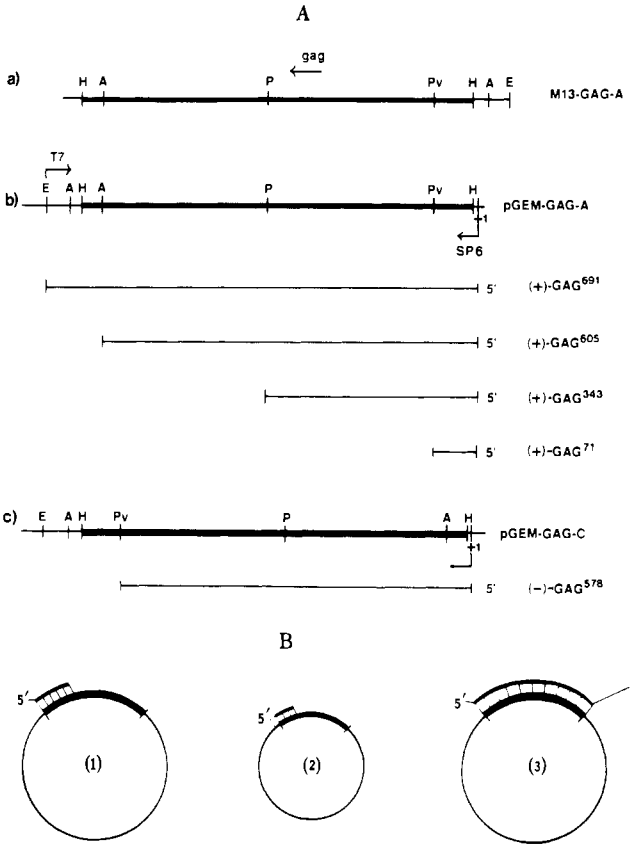

FIGURE 1: Construction of the RNA·DNA hybrids. Panel A: Restriction maps and nomenclature of the M13 (a) and pGEM (b and c) *gag* subclones. The solid line represents the 627-bp HIV-1 *gag* *Hin*dIII (H) insert common to all vectors. (a) The (+)-strand *gag* transcripts (+)-GAG^{71}, (+)-GAG^{343}, (+)-GAG^{605}, and (+)-GAG^{691} were produced by SP6 runoff transcription of the pGEM-GAG-A plasmid that had been linearized by digestion with *Pvu*II (Pv), *Pst*I (P), *Acc*I (A), and *Eco*RI (E), respectively. (b) The (−)-strand *gag* transcript (−)-GAG^{578} was produced by SP6 runoff transcription of pGEM-GAG-C that had been linearized by digestion with *Pvu*II. The superscripts denote the lengths of the corresponding (+/−)-*gag* transcripts. The SP6 transcriptional initiation site (designated +1) within the pGEM-GAG-A and pGEM-GAG-C subclones is indicated by an arrow and is located 8 bp upstream of the *Hin*dIII junction between the vector and the 627-bp *gag* insert. Panel B: Structures of the hybrids. The solid lines represent the *gag* sequence, and the base-paired and mismatched regions of the hybrids are indicated. (1) 5′-Mismatched hybrid, (+)-GAG^{71}/ss-M13-GAG-A; (2) fully complementary hybrid, (+)-GAG^{71}/ss-pGEMf(−)-GAG-A; (3) 5′- and 3′-mismatched hybrid, (+)-GAG^{691}/ss-M13-GAG-A.

However, the relationship between observed p66 clearance size and the amount of HIV-1 RT loaded suggested that low levels of p51 activity (<10% of p66) may have been undetectable by this technique (data not shown). To compare the sensitivity of the gel assay for *E. coli* RNase H vs HIV-1 RT, the relative specific activities of the two enzymes were determined under gel assay conditions by using the DE81 filter binding assay, which specifically measures the rate of formation of small RNA oligonucleotide products from a large RNA·DNA hybrid substrate (data not shown). Under these conditions, the molar specific activity of *E. coli* RNase H activity was approximately 170-fold higher than that of HIV-1 RT. If the activated gel assay paralleled the filter assay in sensitivity and in linearity with respect to time and enzyme concentration, then clearance zones of comparable size would be predicted from 45 μg of HIV-1 RT (p66 position) and 0.04 μg of *E. coli* RNase H, assuming negligible p51-associated RNase H activity of HIV-1 RT, a monomeric molecular mass of 17.5 kDa for *E. coli* RNase H (Kanaya & Crouch, 1983), and equiv-

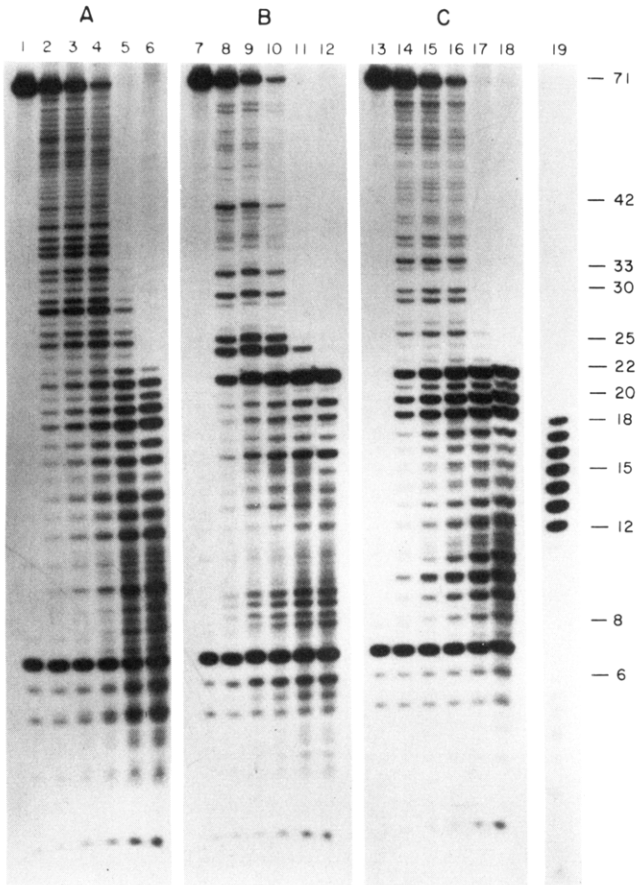

FIGURE 2: Comparative denaturing gel electrophoretic analysis of time course of RNase H cleavage products of uniformly labeled (+)-GAG^{71}/ss-M13-GAG-A hybrid formed by *E. coli* RNase H (panel A), HIV-1 RT (panel B), and M-MuLV RT (panel C; Mn^{2+}-dependent) cleavage. Reactions containing 25 nM hybrid and either 0.19 nM *E. coli* RNase H, 34 nM HIV-1 RT, or 7 nM M-MuLV RT were incubated as described under Materials and Methods. Time points were quenched after 0 (lanes 1, 7, and 13), 2 (lanes 2, 8, and 14), 5 (lanes 3, 9, and 15), 10 (lanes 4, 10, and 16), and 30 min (lanes 5, 11, and 17). The enzyme concentrations of the remaining reactions were then increased ca. 3-fold by the addition of a fresh aliquot of enzyme, and the mixtures were quenched after incubation for a further 30 min (lanes 6, 12, and 18). Samples were electrophoresed as described. The indicated sizes of the 5′-*tri*phosphorylated RNA products are only approximate since they were estimated on the basis of electrophoretic mobility relative to the 5′-*mono*phosphorylated $p(dT)_{12-18}$ marker (lane 19).

alent renaturation and diffusion of the two enzymes in situ. However, a direct comparison of the sizes of the clearance zones from 45 μg of HIV-1 RT vs 0.05 and 1.0 μg of *E. coli* RNase H, respectively, suggested that the sensitivity of the assay was ca. 20–30-fold lower for *E. coli* RNase H than for HIV-1 RT, given the caveats noted above (data not shown).

Gel Electrophoretic Analysis of RNase H Products. (*1*) *5′-Mismatched Hybrids.* The RNase H cleavage products of (+)-GAG^{71}/ss-M13-GAG-A generated by *E. coli* RNase H, HIV-1 RT, and M-MuLV RT were analyzed by denaturing gel electrophoresis. For this comparative analysis, the enzyme concentrations were adjusted to give approximately equal rates of digestion of (+)-GAG^{691}/ss-M13-GAG-A, as measured by the DE81 filter assay (data not shown). As shown in Figure 2, the time-dependent product distributions of the three reactions all differed considerably from one another. Of particular significance was the marked difference between the product distributions generated by *E. coli* RNase H vs HIV-1 RT, suggesting that the retroviral enzyme rather than an *E. coli* contaminant was responsible for the observed HIV-1 RT catalyzed hydrolysis (Figure 2, panel B). The bacterial enzyme generated a large and fairly random distribution of products, as opposed to the retroviral enzymes, which produced a few prominent intermediates of 24–42 nt in size with relatively little accumulation of larger products (>43 nt).

The difference in divalent metal ion dependence of the RNase H activities of *E. coli* and M-MuLV RT was used to confirm that the commercial preparation of recombinant M-MuLV RT used to generate the Mn^{2+}-dependent time course (Figure 2, panel C) was free of contaminating *E. coli* RNase H. Insignificant Mn^{2+}-dependent degradation of (+)-GAG^{71}/ss-M13-GAG-A hybrid by *E. coli* RNase H was observed relative to the rapid Mg^{2+}-dependent cleavage; conversely, the RNase H activity of M-MuLV RT was greater with Mn^{2+} than Mg^{2+}, in accordance with the data of Roth et al. (1985) on the metal ion dependence of the RT activity of this enzyme (data not shown). In conjunction, these results suggested that the M-MuLV RT time course shown in Figure 2 (panel C) was indeed the result of hydrolysis by the RT-associated activity.

To further characterize the products of the RNase H cleavage of the (+)-GAG^{71} RNA, the HIV-1 RT catalyzed degradation of (+)-GAG^{71}/ss-M13-GAG-A was analyzed by using 5′-end-labeled (+)-GAG^{71}, which was synthesized by SP6 RNAP transcription using [γ-^{32}P]GTP. The comparative HIV-1 RT dependent time courses observed by using uniformly labeled vs 5′-end-labeled substrate are illustrated in Figure 3. All of the prominent intermediates (I–V) and several smaller end products (VI–XV) were common to both time courses, confirming that these oligonucleotides were derived from the 5′-mismatched end of the (+)-GAG^{71} RNA. The most noticeable difference in the time courses was the appearance of a smear of small products (4–15 nt) that was only formed by digestion of the uniformly labeled substrate (panel A) and therefore represented a mixture of 3′-derived oligonucleotide products.

(*2*) *Fully Complementary Hybrids.* A fully complementary substrate was prepared by hybridizing uniformly labeled (+)-GAG^{71} to ss-DNA isolated from the phagemid subclone pGEMf(−)-GAG-A. The HIV-1 RT catalyzed hydrolysis of this substrate was compared to that of a related 5′-mismatched substrate to evaluate the effect of the 7-nt mismatch on the product distribution (Figure 4; panel A vs panel B). Removal of the 5′-terminal mismatch significantly altered the cleavage pattern with respect to the size and abundance of intermediate and end products. As shown in Figure 4, the products I and V–XXII were common to both time courses, whereas XXIII–XXVIII were unique to the fully complementary substrate. The final products formed from the fully complementary hybrid consisted of a nonuniform distribution of oligonucleotides ranging in size from 4 to 15 nt (Figure 4, lane 13), of which XII, XV, XX, XXII, and XXV–XXVIII were the most abundant.

(*3*) *Hybrids Bearing 5′- and 3′-Mismatches.* The HIV-1 RT catalyzed digestion of the (+)-GAG^{691}/ss-M13-GAG-A hybrid, which had 3′- and 5′-mismatches of 51 and 7 nt, respectively, resulted in the formation of a prominent cluster of 65–70-nt end products (data not shown). A comparison with the hydrolysis products of the related 3′-terminal truncated substrates (+)-GAG$^{71-605}$ (Figure 1) confirmed that this cluster was unique to the (+)-GAG^{691} substrate and therefore represented oligonucleotides that originated from the unhybridized 3′-terminus of (+)-GAG^{691}.

Discussion

The retrovirally encoded RNase H is the activity responsible for catalyzing complete degradation of the RNA template,

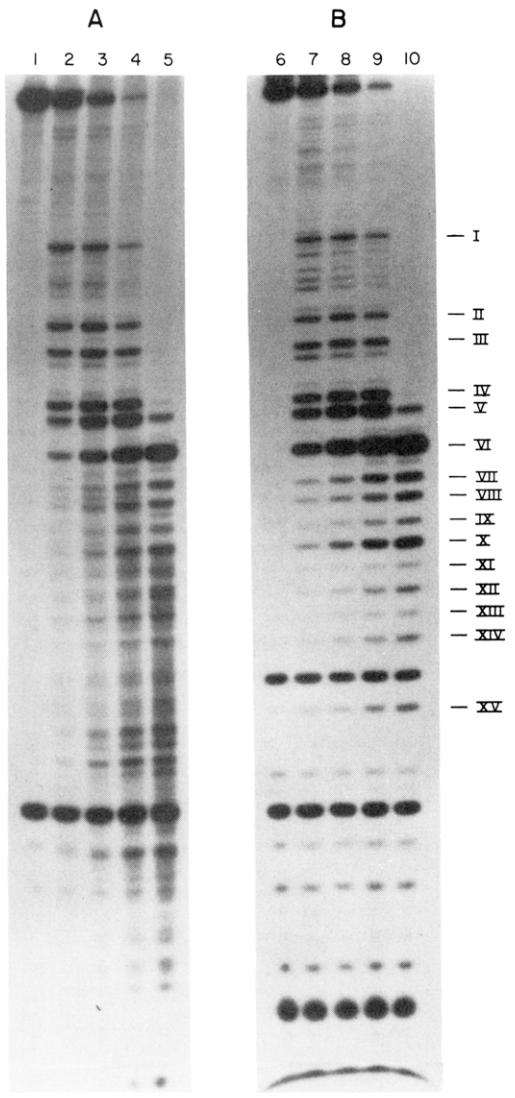

FIGURE 3: Identification of the origin of the RNase H products of HIV-1 RT using a 5′-end-labeled substrate. Reactions containing 25 nM (+)-GAG^{71}/ss-M13-GAG-A substrate in which the RNA was uniformly labeled (panel A) or 5′-end-labeled (panel B) were incubated with 34 nM HIV-1 RT and were quenched after 0 (lanes 1 and 6), 2 (lanes 2 and 7), 5 (lanes 3 and 8), 10 (lanes 4 and 9), and 30 min (lanes 5 and 10) and electrophoresed as described. The products I–XV are referred to in the text.

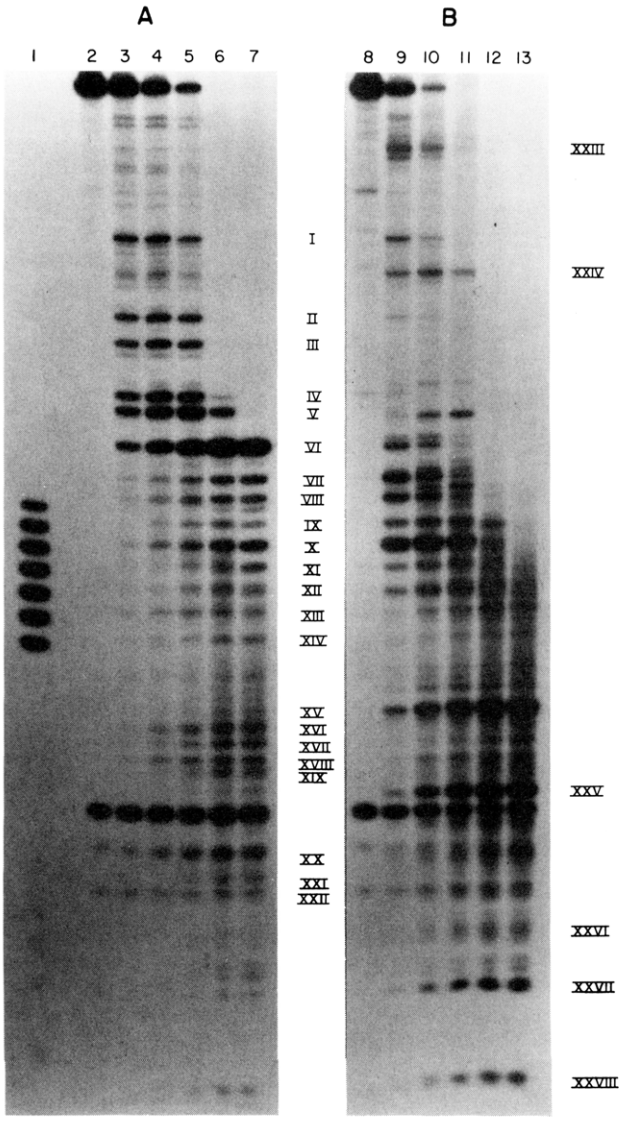

FIGURE 4: Effect of a 5′-mismatch on the RNase H cleavage pattern of HIV-1 RT. Reactions containing 25 nM 5′-mismatched (7-nt) uniformly labeled hybrid (+)-GAG^{71}/ss-M13-GAG-A (panel A), or 25 nM fully complementary hybrid (+)-GAG^{71}/ss-pGEMf(−)-GAG-A (panel B), were incubated with 34 nM HIV-1 RT and quenched after 0 (lanes 2 and 8), 2 (lanes 3 and 9), 5 (lanes 4 and 10), 10 (lanes 5 and 11), 30 (lanes 6 and 12), and 60 min (lanes 7 and 13; as described in the legend to Figure 2). Lane 1: 5′-End-labeled p(dT)$_{12-18}$ size marker. Samples were electrophoresed as described. The products I–XXVIII are referred to in the text.

tRNA primer release, and (+)-strand primer production and subsequent excision during the course of conversion of genomic ss-RNA into viral ds-DNA (Omer & Faras, 1982; Champoux et al., 1984; Smith et al., 1984a,b; Resnick et al., 1984; Hershey & Taylor, 1987; Panganiban & Fiore, 1988). The ability to study the mechanism and to design or discover specific inhibitors of this diverse range of reactions requires the availability of suitable RNA·DNA hybrid substrates. To this end, a simple and general method for the large-scale production of homogeneous RNA·DNA hybrids has been developed. The method is based on the production of runoff RNA transcripts of defined length and sequence utilizing the highly efficient phage SP6 RNA polymerase/promoter system (Krieg & Melton, 1988), followed by hybridization to ss-DNA derived from either a complementary M13 (Messing, 1983) or a phagemid subclone (Mead & Kemper, 1987). This method is superior in both yield and product homogeneity to those previously described for the polynucleotide phosphorylase catalyzed production of homopolymeric hybrids of heterogeneous size (Gorecki & Panet, 1978), and for the synthesis of heteropolymers by the relatively inefficient transcription of ss-DNA templates by *E. coli* RNA polymerase (Srivastava & Modak, 1979).

The hybrids were used to develop an activated gel assay for recombinant p66/p51 HIV-1 RT. The lack of observable RNase H activity of the p51 polypeptide by this technique supports the notion of a modular domain organization of HIV-1 RT analogous to that of M-MuLV RT (Tanese & Goff, 1988), in which the RNase H is entirely located within the COOH-terminal 15 kDa of the p66 polypeptide (Hansen et al., 1988; Tisdale et al., 1988). However, this interpretation not only assumes equivalent renaturation of both polypeptides in situ but is further compromised by the inherently low sensitivity of the activated gel assay. The poor sensitivity of the assay also precluded its use in detecting low levels of contaminating bacterial RNase H in a preparation of *E. coli* expressed HIV-1 RT.

The enzymatic cleavage products of a 5′-mismatched hybrid by HIV-1 RT, M-MuLV RT, and *E. coli* RNase H were

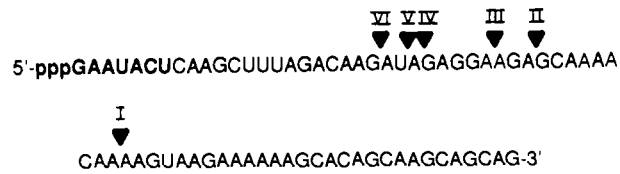

FIGURE 5: Sequence of the (+)-GAG^{71} RNA transcript. The 7-nt 5′-terminal mismatch formed by hybridization of the RNA to ss-M13-GAG-A is highlighted. The approximate positions at which HIV-1 RT cleavage of the 5′-mismatched substrate occurred to yield the 5′-derived products I–VI referred to in the text are indicated.

compared by a high-resolution gel electrophoretic analysis of products. The significant difference between the HIV-1 RT and *E. coli* RNase H product distributions directly confirmed that the preparation of HIV-1 RT used in this study had negligible contamination with *E. coli* RNase H. The large number and size of intermediate products generated by *E. coli* RNase H are in accordance with a distributive endoribonucleolytic mechanism of hydrolysis (Crouch & Dirksen, 1985). In contrast, hydrolysis of the 5′-mismatched substrates by the exoribonuclease retroviral enzymes produced relatively few large intermediates (Figure 2). This difference provided a sensitive and unambiguous method for the detection of contaminating *E. coli* RNase H in recombinant HIV-1 RT, which was particularly useful given the limitations of the activated gel assay.

The bidirectional, partially processive exoribonuclease mechanistic model originally described by Gerard (1981) for AMV and M-MuLV RT was used as a basis for interpreting the RNase H cleavage patterns produced by HIV-1 RT catalyzed hydrolysis of the various hybrid substrates. In terms of this mechanism, a certain fraction of the oligonucleotide products of the 5′-mismatched substrates could be attributed to normal 3′–5′ cleavage from the correctly base-paired 3′-terminus. However, the common ability of HIV-1 RT, M-MuLV RT, and AMV RT to hydrolyze hybrids with both 3′- and 5′-mismatched ends suggests that concomitant hydrolysis from the mismatched 5′-end may have also contributed to the observed product distribution (unpublished results; Gerard, 1981). Indeed, the ability of retroviral RNase H enzymes to excise 5′-mismatched oligonucleotides is consistent with their role in tRNA primer removal (Champoux et al., 1984), as noted by Gerard (1981). Furthermore, the lack of significant accumulation of large intermediates (42–70 nt) from the 5′-mismatched substrates suggested either a partially processive excision of small (5–15-nt) oligonucleotides from the base-paired 3′-end of the hybrid or a direct excision of larger oligonucleotides (25–30 nt) from the 3′- and/or 5′-ends to form primary hydrolysis products that were subsequently degraded further, as was previously observed for M-MuLV and AMV RT (Gerard, 1981). A clearer distinction between these two models would require a product analysis using 3′-end-labeled substrates, which was not included in this study.

The intermediates I–V observed in the course of HIV-1 RT catalyzed cleavage of the 5′-mismatched hybrid (+)-GAG^{71}/ss-M13-GAG-A were all derived from the 5′-end of the substrate (Figures 3 and 5). The accumulation of these intermediates can be explained in several ways. For instance, the (+)-GAG^{71} RNA sequence shown in Figure 5 may contain RNase H "hot spots", at which cleavage occurs at a high frequency to yield relatively few primary products. Alternatively, the intermediates may represent "pause site" products for which further processive hydrolysis is disfavored by either relatively high rates of enzyme dissociation or intrinsically slow kinetics of cleavage. A comparison of the RNase H products of HIV-1 RT and M-MuLV RT indicated significant differences in the distribution of both intermediates and final products, suggesting that although the two enzymes may share the same general retroviral RNase H mechanism (Gerard, 1981), they differ significantly in the sequence dependence of their kinetics of hydrolysis.

Removal of the potentially obstructive 5′-mismatched portion of the (+)-GAG^{71} hybrid resulted in a significant alteration of the HIV-1 RT product distribution, by presumably facilitating concomitant bidirectional 5′–3′ and 3′–5′ hydrolysis to yield a final distribution of 3–15-mer products. The nonuniform distribution of final products suggested a degree of sequence selectivity in the hydrolysis reaction, resulting in the preferential formation and subsequent accumulation of several major products (e.g., XV and XXV). This observation provides a model for rationalizing the specificity of initiation of (+)-strand viral DNA synthesis, in which the PPT oligonucleotide may correspond to a major RNase H product that is resistant to further RNase H and is selected as the primer by virtue of its abundance and relatively large size (Smith et al., 1984a,b; Resnick et al., 1984; Champoux et al., 1984).

In conclusion, the RNase H activity of HIV-1 RT has been found to share general mechanistic features with other retroviral enzymes, in accordance with a common role for the RT-associated RNase H in the replication of retroviruses. At a more detailed level, the use of homogeneous RNA·DNA hybrid substrates allowed the detection of subtle, yet significant differences in the sequence selectivity associated with the RNase H cleavage reactions of HIV-1 RT and M-MuLV RT. The assay systems described herein should be particularly useful for the enzymatic characterization of point and deletion mutants targeted at the putative RNase H domain of HIV-1 RT (Hizi et al., 1988; Tisdale et al., 1988).

Added in Proof. Reports on the RNase H activity of viral HIV-1 RT associated RNase H by Starnes and Cheng (1989) and by Krug and Berger (1989) were published after submission of this paper.

ACKNOWLEDGMENTS

I am particularly grateful to C. Debouck for initiating this work, many helpful suggestions, and assistance with the manuscript. I also thank G. Lazarus for assistance, helpful discussions, and critical evaluation of the manuscript, T. Francis, R. Mayer, M. Rosenberg, P. Hensley, C. Meyers, S. Goff, N. Tanese, and S. Benkovic for helpful suggestions, R. C. Gallo for the BH10 clone, and M. Daniels for excellent technical assistance.

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