Characterization of the p68/p58 Heterodimer of Human Immunodeficiency Virus Type 2 Reverse Transcriptase†

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ABSTRACT: Recently we demonstrated that the p58 subunit of p68/p58 HIV-2 reverse transcriptase (RT) heterodimer, produced by processing of p68/p68 homodimer with recombinant HIV-2 protease, terminates at Met_{484} [Fan, N., *et al*. (1995) *J. Biol. Chem. 270,* 13573−13579]. Here we describe purification and characterization of the p68/p58 heterodimer of recombinant HIV-2 RT. It exhibited both RT and RNase H activities, obeyed Michaelis−Menten kinetics, and was competitively inhibited by the DNA chain terminator ddTTP ($K_{i[app]} = 305 \pm 20$ nM). The HIV-2 RT-associated RNase H exhibited a marked preference for RNA hydrolysis from a HIV-1 *gag*-based heteropolymeric RNA/DNA hybrid in the presence of either Mg^{2+} or Mn^{2+}, compared to the [^{3}H]poly(rA)·poly(dT) or [^{3}H]poly(rG)·poly(dC) homopolymeric substrates. Relative to HIV-1 RT, the RNase H activity of HIV-2 RT was only 5% toward the [^{3}H]poly(rA)·poly(dT) in the presence of Mg^{2+}. The size distribution of products generated from [^{3}H]poly(rA)·poly(dT) by HIV-2 RT-associated RNase H was markedly distinct from that of HIV-1 RT in the presence of Mg^{2+} or Mn^{2+}. The p68/p58 HIV-2 RT heterodimer, produced by specific cleavage using HIV-2 protease, should be useful for inhibition and biophysical studies aimed at discovering and designing drugs directed toward HIV-2.

The reverse transcriptase (RT)[1] of human immunodeficiency virus type 1 (HIV-1) is a well-known target for therapeutic intervention of AIDS (Chandra *et al*., 1985; Mitsuya *et al*., 1990). A second human retrovirus, HIV-2, is also associated with dysfunction of the immune system (Clavel *et al*., 1986a,b). The genomic organization of HIV-2 is similar to that of HIV-1 (Guyader *et al*., 1987; Zagury *et al*., 1988). There is a fairly high (60−70%) homology in the regions of the *pol* gene coding for RT in HIV-1 and HIV-2 (LeGrice *et al*., 1988). Like the RNA-dependent DNA polymerase (RT) and RNase H functions of HIV-1 RT required during viral replication (Goff, 1990), the HIV-2 RT also possesses both the RT and RNase H activities (Hizi *et al*., 1991a). However, the RT enzymes of the two human retroviruses also reveal nonconserved amino acids at some sites and this has been reflected in the insensitivity of HIV-2 RT to the HIV-1 RT-specific inhibitors (Romero *et al*., 1991; Shaharabany & Hizi, 1992; De Clercq, 1994).

It has been shown that HIV-2 RT, like HIV-1 RT, is present in virus particles as a heterodimer consisting of two polypeptides which were tentatively assigned molecular masses of 68 and 54 kDa (DeVico *et al*., 1989). One way to generate heterodimers of recombinant HIV-2 RT is to express the protein in *Escherichia coli* and then depend upon the proteolysis during isolation and purification by endogenous proteases (Warren *et al*., 1992). However, based on studies with HIV-1 RT, the protein recovered from the host expression system is usually heterodimeric with a heterogeneous C-terminus of the smaller subunit (Lowe *et al*., 1988). Another strategy to obtain purified heterodimer is to coexpress the two subunits on a single plasmid (Muller *et al*., 1991), and this requires a knowledge of the exact C-terminus of the smaller subunit. Thus, it is desirable to obtain heterodimeric HIV-2 RT in which the smaller subunit is generated by specific processing of the larger subunit with the homologous HIV-2 protease. This strategy has been successfully applied to generate heterodimeric (p66/p51) HIV-1 RT in which the HIV-1 protease cleaves one of the protomers at the Phe_{440}−Tyr_{441} bond (Chattopadhyay *et al*., 1992).

Recently, we have shown that the p68/p68 homodimer of HIV-2 RT is not cleaved by HIV-2 protease at the Phe_{440}−Tyr_{441} bond, but instead, the HIV-2 protease processes a bond further downstream at Met_{484} (Fan *et al*., 1995a). These findings provided a rationale for the previous observation that the RT heterodimer isolated from HIV-2 lysates was larger than that from HIV-1 (DeVico *et al*., 1989). Also, we concluded that the p68/p58 HIV-2 RT heterodimer, containing the Met_{484}-truncated p58 subunit, is presumably the biologically relevant form of the enzyme *in vivo* (Fan *et al*., 1995a). However, little is known about the biochemical properties of the heterodimeric HIV-2 RT produced by a specific cleavage of p68/p68 homodimer with HIV-2 protease (Fan *et al*., 1995a). The present studies were undertaken to characterize in detail the RT and RNase H activities of this recombinant heterodimeric (p68/p58) HIV-2 RT.

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[1] Abbreviations: HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2; RT, reverse transcriptase; AIDS, acquired immunodeficiency syndrome; IMAC, immobilized metal affinity chromatography; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; RNase H, ribonuclease H; ss-DNA, single-stranded DNA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; dTTP, thymidine triphosphate; dTMP, thymidine monophosphate.

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MATERIALS AND METHODS

Chemicals. General laboratory chemicals were purchased from Sigma Chemical Co. Protein assay reagents, SDS, Tris base, Coomassie Brilliant Blue G-250, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ammonium persulfate, acrylamide, and bisacrylamide were obtained from Bio-Rad Laboratories. Thymidine triphosphate (dTTP), poly(rA)·oligo(dT), poly(dT), and *E. coli* RNA polymerase were obtained from Pharmacia−LKB. [^{3}H]dTTP and [α-^{35}S]ATP were obtained from New England Nuclear Corp. Single-stranded (ss-) DNA−cellulose was from Sigma Chemical Co. Select-D spin column was from 5′−3′, Inc. Recombinant HIV-2 protease was provided by Dr. A. G. Tomasselli. The RNase H substrates [^{3}H]poly(rA)·poly(dT), [^{3}H]poly(rG)·poly(dC), and HIV-1 *gag*-based [^{3}H]RNA/DNA hybrid were prepared as described before (Evans *et al*., 1994).

Recombinant HIV-2 RT Construct. The HIV-2 RT gene (Fan *et al*., 1995a) was cloned from the plasmid SMP10 which contains the HIV-2$_{ROD}$ RT coding region 2381−4095 (HIV-2$_{rod}$ isolate, GenBank Accession Number M15390). In order to facilitate purification of the HIV-2 RT protein by IMAC, an affinity tag (Ala-Leu-hexa-His) was introduced at the N-terminus of the HIV-2 RT. The 5′-primer (CCATCGATGGCTAAACACCACCACCACCACCACCACCAGTCGCCAAAGTAGAG-3′) includes a *Cla*I site, an initiation codon, hexa-His coding region, and 18 bases complementary to the antisense strand of the 5′ end of the RT coding sequences. The 3′-primer (5′-CTTCTCTCATCCGCCAAAACA) matches the sequence in the junction between the antisense strand of the 3′-end of the HIV-2 RT coding region and the pKK223-3 vector. The 1.7-kb HIV-2 RT fragment was amplified by 20 cycles of PCR and purified by low melting point agarose gel. The amplified 1.7-kb RT fragment was then digested with *Cla*I/*Hin*d III and subcloned into the pKK223-3 vector engineered with a *Cla*I/*Hin*dIII site. Competent *E. coli* strain JM 109 was transformed with the construct and selected for ampicillin resistance. Resulting colonies were analyzed by restriction enzyme analysis and screened for expression of the p68 HIV-2 RT protein upon induction with 1 mM IPTG at 0.4 OD$_{600nm}$ as described elsewhere (Sharma *et al*., 1991, 1992). The 5′ hexa-His linker and HIV-2 RT sequence of the resulting HIV-2 RT expression clone were confirmed by DNA sequencing using Sequenase version 2.0 sequencing kit and [^{35}S]dATP.

Processing of p68/p68 Homodimer of HIV-2 RT with HIV-2 Protease and Purification of the p68/p58 Heterodimer. The IMAC-purified p68/p68 homodimer of HIV-2 RT was obtained from crude *E. coli* extracts (Fan *et al*., 1995a). Purified p68/p68 HIV-2 RT (13 mg) was dialyzed overnight in 200 mM Tris, pH 7.4, and 1 M NaCl at 4 °C. This material was then incubated with 0.25 mg of HIV-2 protease and 1.5 mM DTT at 37 °C for 1 h. The reaction mixture was dialyzed overnight in buffer A (50 mM Tris, pH 7.2, 1 mM DTT, 1 mM EDTA, and 6% glycerol) at 4 °C and applied to a 5-mL bed volume ss-DNA−cellulose column equilibrated in buffer A at a flow rate of 0.3 mL/min. The column was washed with 5 column volumes of buffer A until the absorbance returned to baseline. The HIV-2 RT heterodimer was washed using 3 column volumes of 80 mM NaCl in buffer A followed by elution using 3 column volumes of 200 mM NaCl in buffer A (Muller *et al*., 1991). Fractions (0.8 mL) were collected and analyzed for absorbance (280 nm) and protein concentration by the Bio-Rad Bradford protein assay (Bradford, 1976).

SDS−Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis was carried out with 0.75-mm-thick 10% gels as described (Laemmli, 1970) and stained with Coomassie R-250.

HIV-1 gag-Based [^{3}H]RNA/DNA Hybrid. The HIV-1 *gag* gene was derived from the pSMP-15 plasmid which is a Bluescript KS$^+$ (Stratagene) derivative. The 1.4-kb *Sac*I/*Bgl*II DNA fragment from the HIV-1 variant HXB2 (GenBank Accession Number K03455, nucleotides 679−2095) was cloned into the *Sac*I/*Bam*HI site of Bluescript KS$^+$. Purified plasmid DNA was used for *in vitro* transcription performed using a transcription kit from Stratagene and [^{3}H]-ATP and [^{3}H]UTP (DuPont NEN). The DNA template was digested with 1 unit of DNase/μg of DNA for 20 min at 37 °C. The predicted size of the RNA transcripts, purified by standard methods, was 1463 bases. The complementary HIV-1 *gag* DNA strand was also prepared from the pSMP-15 plasmid by PCR. The radiolabeled HIV-1 RNA was hybridized with nonradioactive complementary DNA strands by standard procedures. The HIV-1 *gag*-based [^{3}H]RNA/DNA hybrid was diluted to 50 000 dpm/10 μL and stored at −20 °C.

Enzyme Assays. RNA-dependent DNA polymerase (RT) activity of HIV-2 RT was determined with poly(rA)·oligo(dT) as the template−primer (Fan *et al*., 1995b). For kinetic studies, the concentration of the template−primer was varied between 0.075 and 5 μM. The RNase H activity associated with HIV-2 RT was initially measured using poly(rG)·poly(dC) and poly(rA)·poly(dT), as described earlier for HIV-1 RT (Evans *et al*., 1994). For RNase H kinetic studies, the HIV-1 *gag*-based [^{3}H]RNA/DNA hybrid was used. The reported kinetic parameters (K_m and V_{max}) were mean values ± SD from three independent assays.

Kinetics of ddTTP Inhibition. The inhibition of RNA-dependent DNA polymerase activity of HIV-2 RT was measured by varying the concentration of ddTTP. A nonlinear least-squares program was used for data analysis using an equation derived for competitive inhibition:

$$v = \frac{V_{\text{uninhibited}}}{1 + \dfrac{\text{I}}{K_{\text{i[app]}}}}$$

where

$$K_{\text{i[app]}} = \frac{K_{\text{i}}([\text{dTTP}] + K_{\text{m}})}{K_{\text{m}}}$$

The apparent K_i ($K_{i[app]}$) was determined directly from the nonlinear least-squares computer program. The K_i for ddTTP was calculated by utilizing the dTTP concentration and K_m for dTTP.

Analysis of RNase H Cleavage Products by Denaturing Gel Electrophoresis. The RNase H substrate [^{35}S]poly(rA)·poly(dT) was prepared using *E. coli* RNA polymerase (Volkmann *et al*., 1993). In a total volume of 300 μL, 20 μg of poly(dT) was incubated for 1 h at 37 °C with 50 units of *E. coli* RNA polymerase, 50 nM ATP, and 100 μCi of [α-^{35}S]ATP in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, and 1 mM $MnCl_2$.

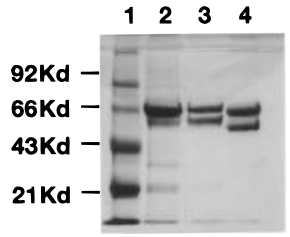

FIGURE 1: SDS−polyacrylamide gel (10%) electrophoresis of p68/p58 HIV-2 RT obtained by HIV-2 protease processing of p68/p68 homodimer followed by purification on a ss-DNA cellulose column. Lane 1, molecular weight markers; lane 2, p68/p68 HIV-2 RT; lane 3, purified p68/p58 heterodimeric HIV-2 RT; lane 4, purified p66/p51 heterodimeric HIV-1 RT.

Table 1: Comparison of RT and RNase H Specific Activities of Homodimer and Heterodimer of HIV-2 Reverse Transcriptase

HIV-2 enzyme	RT activity[a] (units/mg)	RNase H activity[b] (units/μg)
p68/p68	50600 ± 1300	2850 ± 22
p68/p58	86000 ± 1800	4420 ± 57

[a] Determined using poly(rA)·oligo(dT) as described under Materials and Methods. Data represent mean ± SD ($n = 3$). One unit of activity is defined as 1 nmol of [^{3}H]dTMP incorporated into poly(rA)·oligo(dT) in 1 h at 37 °C. [b] Determined using the *gag*-based heteropolymeric RNA/DNA hybrid in the presence of 8 mM Mg^{2+}, pH 8.5. Data represent mean ± SD ($n = 3$). One unit of activity is defined as 1 pmol of TCA-soluble [^{3}H] monomers or oligomers released from the RNA/DNA hybrid in 1 h at 37 °C.

After phenol extraction and ethanol precipitation, the hybrid was dissolved in 100 μL of TE buffer and purified from unincorporated nucleotides by a Select-D spin column. The RNA/DNA hybrid concentration and specific activity were determined by OD_{260nm} absorbance and scintillation counting.

For determination of the degradation products of the HIV-2 RT-associated RNase H, 500 ng of p68/p58 HIV-2 RT or p66/p51 HIV-1 RT in 50 μL of RNase H buffer with either 8 mM $MgCl_2$ or 8 mM $MnCl_2$ were incubated with 2×10^6 cpm (300 ng) of [^{35}S]poly(rA)·poly(dT) substrate at 37 °C. Aliquots (10 μL) of the reaction mixture were removed at 5-, 15-, 30-, and 60-min time points, mixed with 5 μL of formamide-containing stop solution, heat-denatured, and analyzed by electrophoresis on 10% denaturing polyacrylamide gels (Zhan *et al*., 1994).

RESULTS AND DISCUSSION

Enzymatic Characterization of Polymerase Activity of Heterodimeric (p68/p58) HIV-2 RT. Our full-length construct, which contains 574 amino acids, was assigned an apparent molecular mass of approximately 68 kDa (Fan *et al*., 1995a). The smaller subunit of p68/p58 HIV-2 RT heterodimer, produced by HIV-2 protease processing, is referred to as p58. This subunit is 45 amino acids longer than the p51 subunit of HIV-1 RT (Fan *et al*., 1995a). Figure 1 shows the p68/p58 heterodimer purified after *in vitro* processing of p68/p68 homodimer with HIV-2 protease. The purified HIV-2 heterodimer showed a 1:1 distribution of two bands corresponding to relative molecular masses of 68 and 58 kDa (lane 3). Table 1 shows a comparison of RT and RNase H activities of p68/p68 homodimer with the p68/p58 heterodimer. As shown, the homodimer is about 41% and 36% less active than heterodimer with respect to RT and RNase H specific activities, respectively. Thus, HIV-2 RT

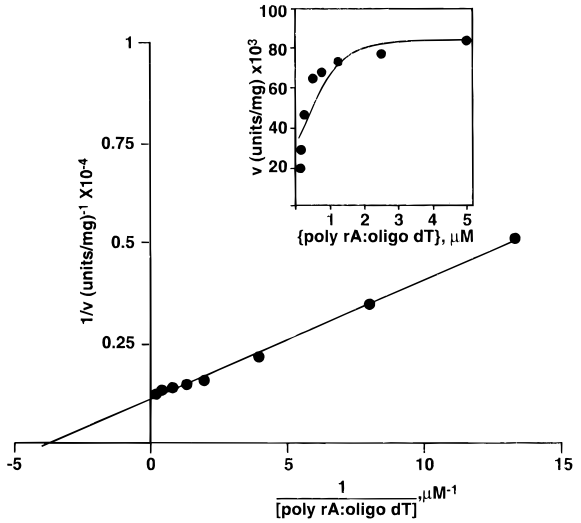

FIGURE 2: Lineweaver−Burk plot showing the effect of varying the concentration of template−primer on RNA-dependent DNA polymerase activity of p68/p58 HIV-2 RT. The second substrate (dTTP) was kept at saturation. (Inset) Michaelis−Menten plot.

heterodimer reported here is enzymatically distinct from the more readily available and previously studied p68/p68 homodimer (Hizi *et al*., 1991b). The p68/p58 heterodimer was used for characterization of its enzymatic properties.

Previously we reported kinetic parameters (K_m and V_{max}) for purified heterodimeric HIV-1 RT by varying the template−primer concentration (Chattopadhyay *et al*., 1992). Figure 2 shows the kinetic parameters (K_m and V_{max}) for heterodimeric HIV-2 RT determined by varying the concentration of the poly(rA)·oligo(dT) and keeping saturating levels of dTTP in the RNA-dependent DNA polymerase activity assay. A straight line was obtained when the data were transformed into a Lineweaver−Burk plot showing $1/v$ versus $1/S$. As shown in the inset in Figure 2, the data fit the Michaelis−Menten equation. These data provide no evidence of nonlinear behavior, substrate inhibition, or allosterism in HIV-2 RT heterodimer kinetics. A K_m value of 0.23 ± 0.02 μM and a V_{max} of 80 000 ± 1656 units/mg were obtained under the defined assay conditions. Similar kinetic analyses were performed for the case where dTTP concentration was varied at subsaturation levels with poly(rA)·oligo(dT) concentration being held at saturation. A similar plot was obtained when the data were fitted into a Michaelis−Menten equation (Figure 3). A K_m value of 4.76 ± 0.41 μM and a V_{max} of 76 500 ± 759 units/mg were obtained under defined assay conditions.

The chain terminator ddTTP is a competitive inhibitor of HIV-1 RT with respect to dTTP (Lowe *et al*., 1991). We have also determined the ability of the chain terminator ddTTP to inhibit RNA-dependent DNA polymerase activity of the wild-type p68/p58 HIV-2 RT for comparison with the p66/p51 HIV-1 RT. Figure 4 shows the theoretical curves for competitive inhibition of HIV-1 RT and HIV-2 RT and the experimental data points for ddTTP inhibition. As shown, the experimental results and theoretical curve calculated for competitive inhibition show good agreement. It was found that ddTTP inhibits HIV-2 RT in a competitive manner with a $K_{i[app]}$ of 305 ± 20 nM and was essentially in the same range as observed for the wild-type p66/p51 HIV-1 RT ($K_{i[app]} = 289 \pm 15$ nM) under defined assay conditions. For HIV-2 RT, K_i for ddTTP inhibition calculated from $K_{i[app]}$

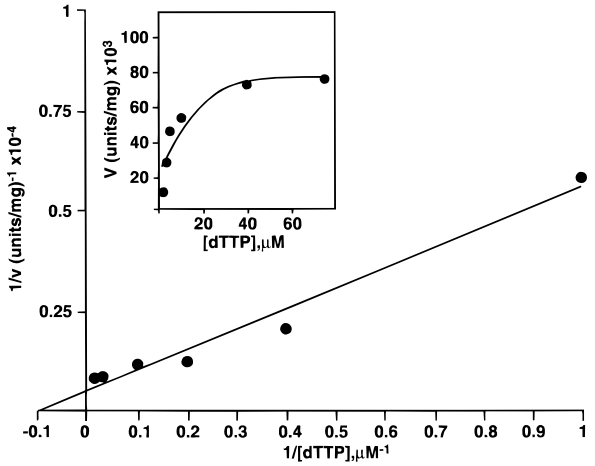

FIGURE 3: Lineweaver−Burk plot showing the effect of varying the concentration of [^{3}H]dTTP while holding the second substrate poly[rA]·oligo[dT] at saturation on the reaction rate of the RNA-dependent DNA polymerase activity of HIV-2 RT.

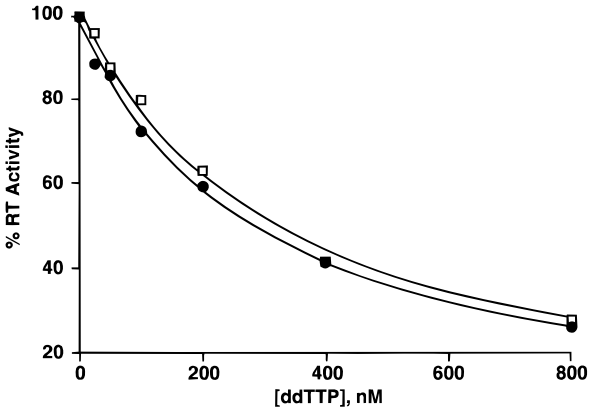

FIGURE 4: Inhibition of the RNA-dependent DNA polymerase activities of HIV-1 RT and HIV-2 RT by ddTTP. □, HIV-2 RT; ●, HIV-1 RT. The activity was assayed by incorporation of [^{3}H]-dTTP into poly[rA]·oligo[dT] as the template−primer. The data were fitted into an equation for competitive inhibition by using a nonlinear least-squares program.

was 17.6 ± 1.1 nM. This value is in agreement with a K_i of 36 nM reported for ddTTP inhibition of HIV-2 RT (Peach *et al*., 1995).

Substrate Preference and Divalent Cation Requirement for RNase H Activity. It has been reported that the RNase H activity of the unprocessed p68 HIV-2 RT is about 10% that of the corresponding p66 HIV-1 RT (Hizi *et al*., 1991b). This comparison was based on studies with the poly(rA)·poly(dT) homopolymeric substrate and Mg^{2+} as the metal ion activator in the RNase H activity assay. Recent studies (Zhan *et al*., 1994) with homopolymeric RNA/DNA hybrids as model substrates have shown that the RNase H activity of HIV-1 RT depends upon substrate composition as well as on the nature of the divalent metal ion. Therefore, we investigated in detail substrate preference and divalent cation requirement for determining RNase H activity of our p68/p58 heterodimeric HIV-2 RT. Results are shown in Table 2 along with the p66/p51 heterodimeric HIV-1 RT included for comparison purposes.

The RNase H activity of HIV-2 RT was <10% of the RNase H activity of HIV-1 RT when poly(rA)·poly(dT) was the substrate and Mg^{2+} was the cation activator. In contrast, with the same substrate, the RNase H activity of HIV-2 RT was 20% that of HIV-1 RT in the presence of Mn^{2+}. With regard to the poly(rG)·poly(dC) hybrid, the RNase H activity of HIV-2 RT was comparable to HIV-1 RT in the presence of Mg^{2+}. However, in the presence of Mn^{2+}, this substrate was relatively less effective with HIV-2 RT than with HIV-1 RT. Again, the RNase H activity was dependent upon the nature of the metal ion activator present in the assay mixture. Notably, the RNase H activity assays used in the above experiments measure the release of TCA-soluble ^{3}H oligonucleotides (<10 nucleotides) from [^{3}H]poly(rA)·poly(dT) (Evans *et al*., 1994).

Heteropolymeric gag-Based RNase H Substrate for HIV-2 RT. Very little is known about the RNase H activity of recombinant HIV-2 RT. In our search for a better RNase H substrate for HIV-2 RT, we studied cleavage of a [^{3}H]-HIV-1 *gag*-based RNA/DNA hybrid used previously for HIV-1 RT-associated RNase H (Evans *et al*., 1994). Table 2 shows a comparison of Mg^{2+}- and Mn^{2+}-dependent RNase H activities of HIV-1 RT and HIV-2 RT in the presence of the HIV-1 *gag*-based RNA/DNA hybrid. The RNase H associated with heterodimeric HIV-2 RT or HIV-1 RT exhibited a marked preference for this HIV-1 *gag*-based RNA/DNA hybrid, irrespective of the nature of the metal ion used in the RNase H activity assays (Table 2). Compared to poly(rG)·poly(dC) substrate, the specific RNase H activity of HIV-2 RT was 300- and 2700-fold higher in the presence of Mg^{2+} and Mn^{2+}, respectively. Interestingly, HIV-1 RT shows Mg^{2+}- and Mn^{2+}-dependent RNase H activities that are approximately 2-fold higher than that for HIV-2 RT with this HIV-1 *gag*-based RNA/DNA hybrid. Taken together, these results show that the HIV-2 RT-associated RNase H activity, to a large extent, depends upon the nature of the RNA/DNA hybrid as well as the divalent cation activator. Also, they demonstrate that the lack of HIV-2 RT-associated RNase H activity (Table 2) with the commonly used [^{3}H]-poly(rA)·poly(dT) substrate is not a reflection of the intrinsic RNase H activity of the HIV-2 RT.

Kinetic Characterization of RNase H Activity of HIV-2 RT. From the above studies it was concluded that the HIV-1 *gag*-based RNA/DNA hybrid is an excellent substrate for studying RNase H activity of HIV-2 RT. Figure 5 shows kinetics of HIV-2 RT-associated RNase H activity at each concentration of the *gag*-based RNA/DNA hybrid in the presence of Mg^{2+}. As shown, the data with this heteropolymeric substrate follows the Michaelis−Menten equation for a uni-substrate condition. A K_m value of 0.42 ± 0.04 μM and a V_{max} of 5380 ± 590 units/μg were obtained from these data under defined assay conditions. These results show that the HIV-1 *gag*-based substrate, as opposed to [^{3}H]poly(rA)·poly(dT), is a suitable substrate for studying activity and inhibition of HIV-2 RT-associated RNase H.

Effect of Divalent Metal Ions on the Size of Products Generated by HIV-2 RT-Associated RNase H. To determine the basis for the relatively low RNase H activity of HIV-2 RT with [^{3}H]poly(rA)·poly(dT) substrate in the presence of Mg^{2+} (Table 2), the size distribution of products generated by RNase H was determined by denaturing gel electrophoresis. As shown in Figure 6 (A and B), the time-dependent size distribution of products, generated by HIV-2 RT RNase H from [^{35}S]poly(rA)·poly(dT) substrate, differed markedly from that of the wild-type HIV-1 RT. For HIV-2 RT, there was absence of significant release of oligonucleotides between 5 and 15 nucleotides in length even after 60 min of incubation (Figure 6B, lanes 2−5). In contrast, under similar

Table 2: Substrate and Metal Ion Preferences for RNase H Activities Associated with Heterodimers of HIV-1 and HIV-2 RT[a]

	specific RNase H activity			
	p66/p51 HIV-1 RT		p68/p58 HIV-2 RT	
RNA/DNA substrate	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
[^{3}H]poly(rA)·poly(dT)[b]	715 ± 71	990 ± 34	36 ± 4	211 ± 12
[^{3}H]poly(rG)·poly(dC)[b]	19720 ± 2930	2185 ± 95	14418 ± 572	847 ± 82
gag-based[c]	9310 ± 140	5400 ± 440	4420 ± 57	2300 ± 66

[a] Data represent mean ± SD ($n = 3$). The Mg^{2+} and Mn^{2+} concentrations were 8 mM. One unit is defined as 1 pmol of TCA-soluble ^{3}H monomers or oligomers released from the RNA/DNA hybrid in 1 h at 37 °C. Other assay details are given under Materials and Methods. [b] The specific activities are expressed in units per milligram. [c] The specific activities are expressed in units per microgram.

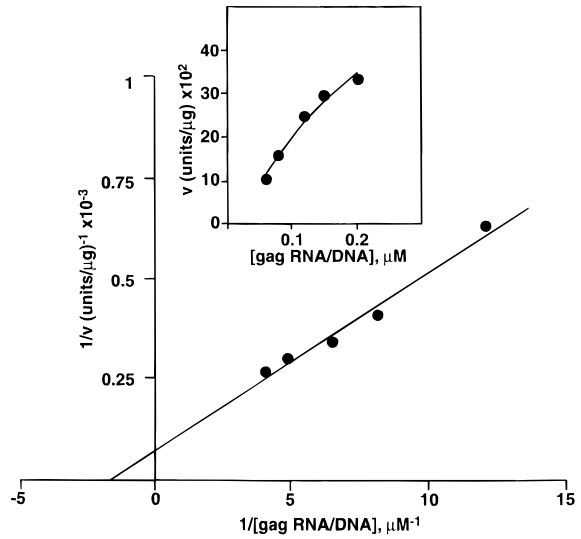

FIGURE 5: Lineweaver−Burk plot showing the effect of varying the concentration of HIV-1 *gag*-based [^{3}H]RNA/DNA hybrid on reaction rate of RNase H activity associated with p68/p58 HIV-2 RT. (Inset) Michaelis−Menten plot.

experimental conditions, the smaller nucleotides were generated by the wild-type HIV-1 RT as early as 5 min of incubation and continued for 60 min (Figure 6A, lanes 2−5). This was consistent with the RNase H activity data (Table 2) in which only the small (<10) oligonucleotides released from the RNA/DNA hybrid can be detected in the RNase H activity assay. Thus the differences observed here are consistent with the dissimilarities observed in the RNase H activities between HIV-2 RT and HIV-1 RT in the presence of poly(rA)·poly(dT) and Mg^{2+} (Table 2).

Interestingly, no mononucleotide was generated in significant amounts even after 60 min (Figure 6A), suggesting that endonuclease activity is responsible for the hydrolysis of the poly(rA)·poly(dT) substrate in the presence of Mg^{2+}. Furthermore, our data support the view that the endonuclease RNase H activity of the HIV-2 RT enzyme, compared to that of HIV-1 RT, is significantly decreased for this substrate under defined conditions. This is consistent with the reported (Hizi *et al*., 1991b) low (10%) Mg^{2+}-dependent RNase H activity of p68 HIV-2 RT compared to HIV-1 RT in the presence of poly(rA)·poly(dT) substrate.

The cleavage products from [^{35}S]poly(rA)·poly(dT) by HIV-2 RT RNase H in the presence of Mn^{2+} differed from those shown above when Mg^{2+} was the metal ion activator. As shown in Figure 6 (C and D), smaller oligonucleotides (5−10 nucleotides in length) were produced very early in the reaction (lanes 2−5), in agreement with a recent report (Zhan *et al*., 1994). It appears that both endonuclease and exonuclease activities participate in the RNA degradative activity in the presence of Mn^{2+} (Figure 6C,D). This interpretation is consistent with the presence of both large and smaller oligonucleotides at early time points. In the presence of Mn^{2+}, the relative rate of formation of these smaller (<10 nucleotides) products by HIV-2 RT was rather slow compared to HIV-1 RT. The relative abundance of intermediates (10−15 oligonucleotides in length) observed between 15 and 60 min for HIV-2 RT (Figure 6D, lanes 2−5) suggests that perhaps its exonuclease activity, compared to that of HIV-1 RT, is relatively less efficient in the presence of Mn^{2+}. These results led us to conclude that *in vitro* RNase H activity of the HIV-2 RT heterodimeric enzyme depends upon the composition of the hybrid substrate as well as the nature of the divalent cation activator.

Studies with immunoaffinity-purified HIV-2 RT from viral lysates show that both the subunits of the heterodimeric HIV-2 enzyme are slightly larger than their HIV-1 counterparts (DeVico *et al*., 1989). Our results on purified recombinant HIV-2 RT (Figure 1), obtained by *in vitro* processing of p68/p68 HIV-2 RT with HIV-2 protease, are in complete agreement with the data for HIV-2 lysates. These results are consistent with *in vitro* studies demonstrating that the differential processing of homodimers of reverse transcriptases from HIV-1 and HIV-2 is a consequence of the distinct specificity of the viral proteases (Fan *et al*., 1995a).

There are conflicting reports (Hizi *et al*., 1991b; Warren *et al*., 1992) on the RNase H activity of HIV-2 RT. In these reports, the HIV-2 RT was not produced by specific cleavage with the homologous HIV-2 protease. In contrast to these studies, our results shed light on the RNase H activity of the biologically relevant form of p68/p58 HIV-2 RT. The p68/p58 HIV-2 RT possesses high Mg^{2+}- and Mn^{2+}-dependent specific RNase H activity with the HIV-1 *gag*-based heteropolymeric substrate. Although both HIV-1 RT and HIV-2 RT possess RNase H activity, they appear to differ significantly in the relative activities with poly(rA)·poly(dT) and poly(rG)·poly(dC) substrates in the presence of Mg^{2+} and Mn^{2+}, respectively (Table 2 and Figure 6). Despite the fact that the two proteins are 60% identical at the amino acid level, they show a large difference in their content of charged amino acids (Ratner *et al*., 1985). Thus, the interaction of the enzyme with the RNA/DNA hybrid seems to be partly influenced by the composition and/or structure of the substrate. Moreover, the additional use of a heteropolymeric RNA/DNA hybrid has allowed us to delineate the effects of substrate composition and divalent metal ions on RNA hydrolysis by HIV-2 RT.

A number of factors led us to characterize p68/p58 HIV-2 RT obtained from p68/p68 homodimer by the action of HIV-2 protease. The reasons were that (a) purified p68/p68 homodimer can be prepared easily from crude *E. coli*

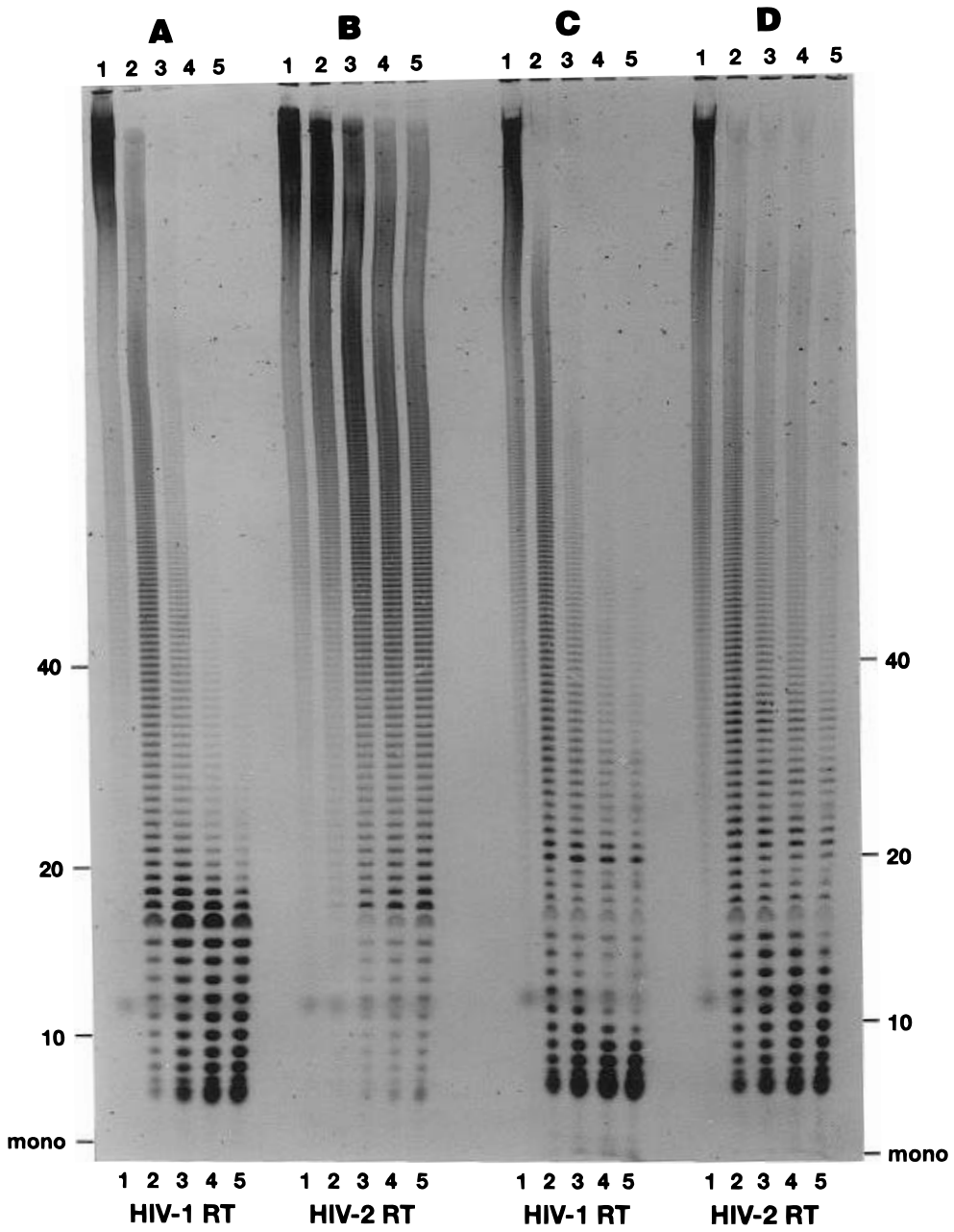

FIGURE 6: Determination of the sizes of the products of RNase H digestion of [^{35}S]poly(rA)·poly(dT) by HIV-1 RT (panels A and C) and HIV-2 RT (panels B and D). Panels A and B show Mg^{2+}-dependent RNase H activity, while panels C and D show Mn^{2+}-dependent RNase H activity. Lanes 1−5 represent 0-, 5-, 15-, 30-, and 60-min time points of incubation at 37 °C. The approximate sizes of RNA cleavage products are indicated.

extracts, (b) purified recombinant HIV-2 protease was readily available, (c) the heterodimer RT produced by processing with HIV-2 protease could be easily purified by affinity chromatography on ss-DNA−cellulose, (d) on the basis of our most recent work (Fan *et al*., 1995a) we were confident that the natural COOH terminus in the p58 subunit should be the result of cleavage by the HIV-2 protease at the previously characterized cleavage of the Met_{484}−Ala_{485} bond, and (e) the enzymatic properties of p68/p58 HIV-2 RT can be directly compared with the already available p66/p51 HIV-1 RT produced by using an identical strategy (Chattopadhyay *et al*., 1992).

It is well-known that addition of a hexahistidine affinity tag or other amino acid residues at the N-terminus of HIV-1 RT has no effect on the polymerase function (Prasad & Goff, 1989; LeGrice & Gruninger-Leitch, 1990; Sharma *et al*., 1991; Chattopadhyay *et al*., 1992). The regions of the *pol* gene coding for RT from HIV-1 and HIV-2 show that HIV-2 RT exhibits 60% sequence homology with HIV-1 RT (Guyader *et al*., 1987). In fact, the polymerase active site for both the enzymes is present in the amino acid region 180−190 (Tong *et al*., 1993). Therefore, it is logical to assume that the N-terminal hexahistidine tag would have no deleterious effect on the polymerase function of HIV-2 RT.

Our results show that an HIV-1 *gag*-based RNA/DNA hybrid is an excellent substrate for the RNase H activity of HIV-2 RT. This finding has allowed us to demonstrate that RNase H kinetics of HIV-2 RT can be conveniently followed with this *gag*-based hybrid. To our knowledge, this is the first report on purification and characterization of polymerase and RNase H activities of the heterodimeric (p68/p58) form of HIV-2 RT. This well-characterized, homogeneous prepa-

ration of p68/p58 HIV-2 RT heterodimer should be suitable for inhibition and cocrystallization studies aimed at discovering and understanding drugs directed toward the replicative machinery of HIV-2.

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