

# Duplex structural differences and not 2'-hydroxyls explain the more stable binding of HIV-reverse transcriptase to RNA-DNA versus DNA-DNA

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## ABSTRACT

**Human immunodeficiency virus reverse transcriptase (HIV-RT) binds more stably in binary complexes with RNA-DNA versus DNA-DNA. Current results indicate that only the -2 and -4 RNA nucleotides (-1 hybridized to the 3' recessed DNA base) are required for stable binding to RNA-DNA, and even a single RNA nucleotide conferred significantly greater stability than DNA-DNA. Replacing 2'-hydroxyls on pivotal RNA bases with 2'-O-methyls did not affect stability, indicating that interactions between hydroxyls and RT amino acids do not stabilize binding. RT's  $K_d$  ( $k_{off}/k_{on}$ ) for DNA-DNA and RNA-DNA were similar, although  $k_{off}$  differed almost 40-fold, suggesting a faster  $k_{on}$  for DNA-DNA. Avian myeloblastosis and Moloney murine leukemia virus RTs also bound more stably to RNA-DNA, but the difference was less pronounced than with HIV-RT. We propose that the H- versus B-form structures of RNA-DNA and DNA-DNA, respectively, allow the former to conform more easily to HIV-RT's binding cleft, leading to more stable binding. Biologically, the ability of RT to form a more stable complex on RNA-DNA may aid in degradation of RNA fragments that remain after DNA synthesis.**

## INTRODUCTION

During HIV-1 replication, the role of its reverse transcriptase (HIV-RT)<sup>1</sup> in binding both DNA and RNA substrates is critical, ultimately, for the production of double-stranded viral DNA that can be integrated into the host genome. This process is largely achieved as a result of the multifunctional nature of the enzyme, specifically its ability to synthesize DNA as both a DNA- and RNA-dependent DNA polymerase, as well as RNase H

activity, which is responsible for the degradation of RNA regions of RNA-DNA hybrids (1).

Several studies have provided a more comprehensive examination of the interaction of HIV-RT with DNA and RNA substrates, concluding, among other findings, that the dissociation of the polymerase from DNA-primed RNA templates is much slower than from DNA-primed DNA templates (2–5). Analysis of DNA-DNA and RNA-DNA crystal structures indicate that interactions with RT amino acids are similar and that both templates have essentially the same trajectory through the substrate binding cleft (6–9). Interactions with the DNA primer strands also appear to exhibit a high degree of similarity, as do many interactions with the RNA and DNA template strands. Conversely, a major difference between these hybrids is several additional potential interactions between RT amino acids and RNA template 2'-hydroxyl groups. Nine template nucleotides and 11 amino acids are proposed to be involved in the 2'-hydroxyl interactions, which span the polymerase and RNase H domains of RT. These interactions, especially those near the RNase H active site, may be important for aligning the RNA for RNase H cleavage, while the numerous additional 2'-hydroxyl contacts may contribute, at least in part, to RT's slower dissociation from RNA-DNA (6,10). In addition, DNA-DNA and RNA-DNA hybrids are known to take on different structures with the former generally being B-form and the latter H-form, an intermediate between A- (the form taken by most RNA-RNA hybrids) and B-form (11). These different structures could also play a role in binding.

Recently, using a 50-nt chimeric template that consisted of a region of RNA flanked by DNA at the 3'- and 5'-ends, we showed that the ~20-fold (for the particular template tested) slower dissociation rate constant ( $k_{off}$ ) of HIV-RT from RNA-DNA versus DNA-DNA substrates required just a short, 5-bp RNA-DNA hybrid region (12). Slower dissociation was also contingent upon the RNA being hybridized to the last 5 nt at the 3'-end of a recessed DNA strand. This essentially positions RT's

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polymerase active site and primer grip region directly over the RNA. Only four of the nine postulated 2'-hydroxyls involved in amino acid contacts are within the first five template nucleotides proximal to the primer 3' terminus. If amino acid-2'-hydroxyl interactions are responsible for tighter binding to RNA templates, then interactions outside the polymerase site, including those in the RNase H domain, presumably do not make a significant contribution. While not eliminating the role of some 2'-hydroxyl interactions, specifically those near the polymerase domain, in differential binding, this raises the possibility of a structural explanation.

In this study, we attempted to expand upon these initial findings by modifying the short, 5-bp RNA-DNA hybrid region in an effort to determine what conditions were both necessary and sufficient to confer tighter binding of HIV-RT to RNA-DNA versus DNA-DNA hybrids. Analysis of various chimeric substrates revealed that RT dissociated from substrates containing only two of the five RNA nucleotides at a rate similar to a complete 50-nt RNA. Additionally, replacement of the 2'-hydroxyl groups of the pivotal nucleotides with 2'-*O*-methyl groups did not affect RT binding, indicating that hydrogen bonding between RT amino acids and the RNA nucleotides was not contributing to tight binding. Finally, equilibrium dissociation constants ( $K_d$ ) were comparable for both RNA-DNA and DNA-DNA duplexes, even though the former showed much slower dissociation rates. These results provide strong support for structural differences between RNA-DNA and DNA-DNA duplexes as the reason for the more stable binding to the RNA-DNA. The potential biological relevance of these findings is also discussed.

## MATERIALS AND METHODS

### Materials

Custom oligonucleotides were from Integrated DNA Technologies. Plasmid clone for wild-type HIV-RT (type HXB2) was a kind gift from Dr Michael Parniak (University of Pittsburgh), and was prepared and purified as described (13). T4 polynucleotide kinase (PNK) was purchased from New England Biolabs. PCR grade dNTPs were purchased from Roche Applied Sciences. G-25 spin columns were from Harvard Apparatus. Radiolabeled nucleotides were from Perkin-Elmer. All additional reagents were purchased from Sigma Aldrich, Fisher Scientific or VWR.

### Methods

*Preparation of radiolabeled DNA strands.* Twenty-five picomoles of DNA (31–35 nt in length) was 5'-<sup>32</sup>P-end-labeled using PNK. The labeling reaction was performed at 37°C for 30 min, as per the manufacturer's protocol. Reactions were shifted to 70°C for 15 min in order to heat inactivate the PNK. The DNA was then centrifuged on a Sephadex G-25 column in order to remove any excess radiolabeled nucleotide.

*Preparation of RNA-DNA and DNA-DNA hybrids.* Hybrids were prepared by mixing 2 pmol of 5'-<sup>32</sup>P-labeled DNA from above and 2 pmol of 50-nt template (see 'Results' section for full list of template sequences) in 15 µl of buffer containing 50 mM Tris-HCl pH = 8, 80 mM KCl and 1 mM dithiothreitol (DTT), 0.1 mM EDTA pH = 8. Reactions were placed at 80°C for 5 min and then allowed to slow cool to room temperature prior to use.

*Determination of dissociation rate constants ( $k_{off}$ ) by primer extension.* HIV-RT (20 nM) was preincubated with hybrids (20 nM) in 42 µl of buffer containing 50 mM Tris-HCl pH = 8, 80 mM KCl, 2 mM MgCl<sub>2</sub> and 1 mM DTT for 3 min at room temperature. After preincubation, 8 µl of trap supplement in the same buffer containing heparin sulfate (final concentration 2 µg/µl) was added to each reaction. The purpose of this supplement was to bind and sequester enzyme that had dissociated from substrates. Following trap addition, 5 µl aliquots were removed at 15 s (2 s, 5 s and 10 s, time points were also taken of substrate S2P33-D50), 30 s, 1 m, 1.5 m, 2 m, 2.5 m, 3 m and 4 m or 15 s, 1 m, 2 m, 4 m, 8 m and 16 m for DNA or RNA containing templates, respectively, and added to a tube containing 1 µl of dNTP supplement in reaction buffer plus dNTPs (100 µM final concentration). Tubes were incubated at room temperature for 2 min, then terminated by the addition of 6 µl of 2X formamide gel loading buffer (90% formamide, 20 mM EDTA pH = 8.0, 0.25% xylene cyanol and bromophenol blue). A trap control in which RT was mixed with the heparin trap and dNTPs prior to substrate addition and a full extension control in which trap was excluded were also performed and incubated for 10 min prior to 2X formamide gel loading buffer addition. Since the rate of dissociation for enzyme-nucleic acid substrate complexes is slow in comparison to the rate of polymerization (14,15), the amount of HIV-RT bound at the initiation of each reaction can be analyzed by measuring the amount of primer extension. A  $t = 0$  sample was performed by mixing 1 µl of heparin trap supplement and 1 µl of dNTP supplement together and adding this to 4 µl of the reaction + RT preincubation mix (see above). After 2 min 6 µl of 2X gel loading buffer was added to the sample. All samples were subsequently loaded onto a 12% polyacrylamide/7 M urea sequencing gel and subjected to electrophoresis as previously described (16). Reactions with AMV- or MuLV-RT were conducted in the same manner except both DNA-DNA and RNA-DNA duplexes were run over a 16-min time course and the trap was poly(rA)-oligo(dT<sub>20</sub>) [8:1 rA:dT (w:w), final concentration 0.4 µg/µl] instead of heparin for AMV-RT. Magnesium was omitted from the preincubations in some experiments which were conducted with 20 rather than 80 mM KCl.

*Determination of equilibrium dissociation constants ( $K_d$ ) by primer extension.* HIV-RT (0, 0.5, 1, 2, 3, 4, 6 and 8 nM) was preincubated with hybrids (2 nM) in 8 µl of buffer containing 50 mM Tris-HCl pH = 8, 80 mM KCl, 2 mM MgCl<sub>2</sub> and 1 mM DTT for 3 min at room temperature. After preincubation, 2 µl of trap supplement in the same

buffer containing dNTPs (final concentration 100  $\mu$ M) and heparin sulfate (final concentration 2  $\mu$ g/ $\mu$ l) was added to each reaction. The purpose of this supplement was to bind and sequester enzyme that had dissociated from substrate. Tubes were incubated at room temperature for 2 min, then terminated by the addition of 10  $\mu$ l of 2X formamide gel loading buffer. A trap control and full extension control were performed as described above for the  $k_{\text{off}}$  determinations. Samples were processed as described for the  $k_{\text{off}}$  determinations. Reactions with AMV-RT were conducted in the same manner except the trap was poly(rA)-oligo(dT<sub>20</sub>) [8:1 rA:dT (w:w), final concentration 0.4  $\mu$ g/ $\mu$ l] instead of heparin and enzyme amounts were 1, 2, 4, 6, 8, 10 and 12 nM.

*Visualization and quantification of primer extension.* In order to visualize and quantify primer extension, dried polyacrylamide gels were processed on an FLA-5100 or FLA-7000 phosphorimager from Fujifilm Life Sciences. Dissociation rate constants ( $k_{\text{off}}$  values) were obtained by fitting data to a nonlinear least-squares equation for single, exponential decay [ $f(x) = ae^{-bx}$ , where  $a$  is the  $y$ -intercept at time zero and  $b$  is the dissociation rate] using Sigma Plot (Jandel Corp.). Data collected from time,  $t = 0$  was not included in this calculation, as aberrant decreases in primer extension were routinely observed between this and the next measured time point for some substrates (12). Calculation of equilibrium dissociation constants ( $K_d$ ) was determined from graphs of the concentration of extended primer versus concentration of RT by nonlinear least square fit to the quadratic equation:  $[ED] = 0.5([E]t + [D]t + K_d) - 0.5(([E]t + [D]t + K_d)^2 - 4[E]t[D]t)^{1/2}$ , where  $[E]t$  is the total enzyme concentration and  $[D]t$  is the total hybrid concentration (17). Experiments were generally repeated two to four times and averages  $\pm$  standard deviations are reported in Table 1. Note that this approach actually yields an 'apparent  $K_d$ ' value as it is dependent on a secondary measurement (polymerase extension) to assess binding. This would be a concern if there were secondary binding sites on the substrates that could strongly compete with the 3' recessed terminus for RT, or if a substantial proportion of RT interactions with the 3' terminus were nonproductive with RT dissociating before incorporating nucleotides. Each of these concerns is of very low probability for these substrates.

## RESULTS

### RNA–DNA and DNA–DNA substrates used to test HIV-RT binding

In previous work, we showed that RT dissociated  $\sim$ 20 times more slowly from a 33-nt DNA hybridized to a 50-nt RNA template (P33-R50, see Figure 1) as compared to the same DNA hybridized to a homologous DNA template (P33-D50). Progressively reducing the amount of RNA in the template strand showed that only 5 nt of RNA were required for strong binding, as a chimeric template composed of (5'–3') 15 bases of DNA followed by 5 bases of RNA then 30 of DNA (P33-D15R5D30) bound to RT with even greater stably

then P33-R50 (12). In addition, positioning of the 3' terminus of the shorter DNA (P33) was shown to be pivotal to tight binding, as substrates in which the 5-nt RNA–DNA hybrid region were not positioned over the recessed 3' terminus of the DNA dissociated essentially like a homologous DNA template. We therefore concluded that positioning of the 3' terminus directly over the short, 5-bp region of RNA–DNA was both necessary and sufficient for mimicking the more stable interaction of RT with RNA templates. Note that the previous experiments were conducted using the RNase H minus HIV-RT mutant, E478>Q, in the presence of  $Mg^{2+}$ , or wild-type HIV-RT in the absence of  $Mg^{2+}$ . This was required since it is not possible to accurately measure off-rates on an RNA–DNA hybrid with wild-type RT in the presence of  $Mg^{2+}$  due to RNase H activity. Based on its nearly equivalent binding to DNA–DNA substrates in the presence of 4–6 mM  $Mg^{2+}$  and DNA synthetic properties essentially identical to wild-type RT, E478>Q was considered a good model for the wild-type enzyme (18). Although RT substrate interactions in the absence of  $Mg^{2+}$  are considerably less stable than in its presence, the same trend was observed using wild-type HIV-RT in the absence of  $Mg^{2+}$ , with tight binding equivalent to a complete RNA template requiring just the properly positioned 5-bp hybrid region (12).

To examine this 5-bp region more closely, several changes were made in the current report. These included shortening the region and converting each base to DNA, as well as using 2'-*O*-methylated nucleotides to replace some or all of the bases (Figure 1 and Table 1). Given that these substrates are not cleaved by RT's RNase H activity, most experiments were conducted in the presence of 2 mM  $Mg^{2+}$  and 80 mM KCl, using wild-type enzyme to more closely approximate physiological conditions.

### RT dissociates from templates with just two RNA nucleotides at a rate similar to a complete RNA template and even a single RNA nucleotide at the –4 position results in a dissociation rate much slower than a complete DNA template

To determine if all five bases of the RNA–DNA hybrid region noted above were required for slow dissociation of RT, we generated a series of chimeric substrates containing one to five of the RNA nucleotides. These templates (Figure 1, D15R5D30, D15R4D31, D15R3D32, D15R2D33 and D15R1D34) were hybridized to the P33 DNA and the  $k_{\text{off}}$  of RT from each duplex was measured. DNA P33 is designed to position the five base RNA region of D15R5D30 in the polymerase domain of RT with the 5' most RNA base bound to the 3' terminal base of P33. Reverse transcriptase dissociated from the P33-D15R5D30 substrate  $\sim$ 38 times more slowly than to a homologous DNA template (P33-D50, see Table 1). Replacement of the fifth nucleotide (–5 position relative to the recessed 3' terminus of P33) with a DNA base resulted in a small but insignificant increase in the off-rate under the conditions used (Table 1, compare P33-D15R5D30 to P33-D15R4D31, see Figure 2 for a

**Table 1.** Dissociation rate constants ( $k_{\text{off}}$ ) and equilibrium dissociation constants ( $K_d$ ) for RT-substrate complexes

<sup>a</sup> Duplex substrate	<sup>b</sup> Dissociation rate ( $k_{\text{off}}$ ) ( $\text{min}^{-1}$ )	<sup>c</sup> Fold decrease in $k_{\text{off}}$	$K_d$ (nM)
<b>Binding of RT to DNA–DNA duplex</b>			
P33-D50	$0.90 \pm 0.12$	1	$2.7 \pm 1.3$
<b>Effect of shortening the 5-bp RNA–DNA hybrid region in chimeric templates</b>			
P33-D15R5D30	$0.024 \pm 0.006$	38	$1.4 \pm 0.6$
P33-D15R4D31	$0.030 \pm 0.006$	30	
P33-D15R3D32	$0.13 \pm 0.04$	6.9	
P33-D15R2D33	$0.16 \pm 0.04$	5.6	
P33-D15R1D34*	$0.41 \pm 0.07$	2.2	$2.6 \pm 1.3$
<b>Binding of RT to duplex with one or two RNA nucleotides in the template</b>			
P33-D15R1(–1)D34*	$0.41 \pm 0.07$	2.2	$2.6 \pm 1.3$
P33-D16R1(–2)D33	$0.11 \pm 0.02$	8.2	$1.5 \pm 0.3$
P-33-D17R1(–3)D32	$0.25 \pm 0.05$	3.6	
P33-D18R1(–4)D31	$0.066 \pm 0.012$	14	
P33-D16R1(–2)D1R1(–4)D31**	$\leq 0.024$	$\geq 38$	
<b>Effect of changing the nucleotide composition at the pivotal –4 template position</b>			
P33-D18R1(–4U>G)D31	$0.19 \pm 0.05$	4.7	
P33-D18R1(–4U>C)D31	$0.11 \pm 0.02$	8.2	
P33-D18r1(–4U>A)D31	$0.18 \pm 0.04$	5	
<b>Effect of changing the composition at the –2 position</b>			
P33-D16R1(–2U>A)D33	$0.090 \pm 0.006$	10	
P33-D16R1(–2U>C)D33	$0.11 \pm 0.01$	8.2	
<b>Effect of repositioning the pivotal –4 template nucleotide</b>			
P35-D18R1(–4>–6)D31	$0.39 \pm 0.06$	2.3	
P34-D18R1(–4>–5)D31	$0.11 \pm 0.01$	8.2	
P32-D18R1(–4>–3)D31	$0.66 \pm 0.06$	1.4	
P31-D18R1(–4>–2)D31	$0.34 \pm 0.09$	2.6	
<b>Effect of replacing 2'-hydroxyls with 2'-O-methyls</b>			
P33-D18methylR1(–4)D31	$0.042 \pm 0.006$	21	
P33-D15methylR5D30	$0.030 \pm 0.006$	30	$1.6 \pm 1.0$
<b>Analysis of binding to a duplex with an unrelated nucleotide sequence</b>			
S2P33-D50	$9.3 \pm 3.2$	1	
S2P33-D15R5D30	$0.10 \pm 0.03$	93	
S2P33-D18R1(–4)D31	$0.54 \pm 0.02$	17	
S2P33-D18R1(–4A>U)D31	$0.25 \pm 0.01$	37	
S2P33-D16R1(–2)D1R1(–4)D31	$0.12 \pm 0.01$	78	
<b>Binding of AMV- and MuLV-RT to duplex substrates</b>			
AMV, P33-D50	$0.10 \pm 0.02$	1	$2.8 \pm 1.0$
AMV, P33-D15R5D30**	$\leq 0.024$	$\geq 4.2$	$2.1 \pm 0.8$
MuLV-RT, P33-D50	$0.16 \pm 0.02$	1	
MuLV-RT, P33-D15R5D30	$0.078 \pm 0.024$	2.1	
<b>***Binding to 50 nucleotide DNA or RNA templates in the absence of <math>\text{Mg}^{2+}</math></b>			
HIV-RT, P33-D50	$1.3 \pm 0.1$	1	
HIV-RT, P33-R50	$0.048 \pm 0.012$	27	
AMV, P33-D50	$0.96 \pm 0.06$	1	
AMV, P33-R50	$0.43 \pm 0.18$	2.2	
MuLV-RT, P33-D50	$0.33 \pm 0.02$	1	
MuLV-RT, P33-R50	$0.14 \pm 0.05$	2.4	

<sup>a</sup>For an illustration of the duplex sequences and configurations see Figure 1.

<sup>b</sup> $k_{\text{off}}$  or  $K_d$  for HIV-RT on the specific duplexes; see 'Materials and Methods' section for an explanation of how this was determined; results are an average of two to four independent experiments  $\pm$  standard deviation.

<sup>c</sup>All numbers are relative to the specific DNA–DNA duplex of homologous sequence which was set equal to 1; larger numbers indicate slower dissociation of RT and tighter binding.

\*These two substrates are identical

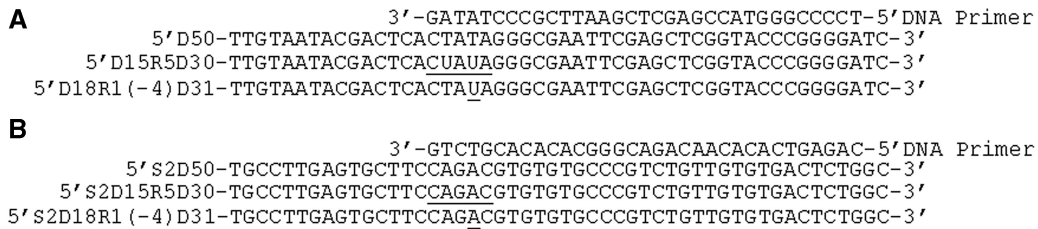
\*\* $k_{\text{off}}$  was too low to be reliably determined under the conditions used; numbers provided indicate that it was at least as low as the lowest measured off-rate in the assays.

\*\*\*Assays were performed without  $\text{Mg}^{2+}$  in dissociation phase and with 20 rather than 80 mM KCl in order to help stabilize binding, which is weaker in the absence of  $\text{Mg}^{2+}$ .

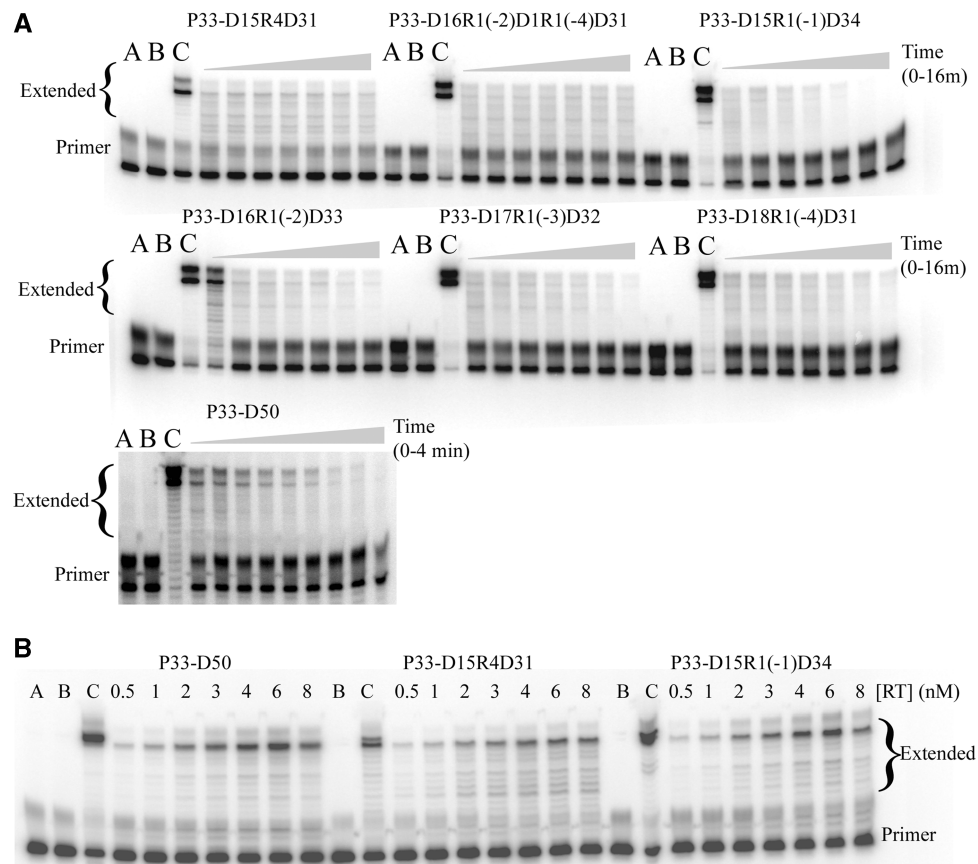
representative experiment used to produce the values in Table 1). In contrast, the  $k_{\text{off}}$  value increased  $\sim 4$ -fold when the –4 and –5 bases were replaced by DNA (P33-D15R3D32). A further, small but insignificant increase was observed when the –3, –4, and –5 nt were replaced (D15R2D33), while a substrate in which only the –1 nt was RNA (P33-D15R1D34) exhibited another 2–3-fold increase in  $k_{\text{off}}$ . From these results, it was clear

that only a 4-bp RNA–DNA region was required for strong binding and that even a single RNA–DNA base pair at the –1 position conferred slower dissociation ( $\sim 2$ – $3$ -fold) as compared to a complete DNA–DNA duplex (P33-D50).

To further test the role of each of the four RNA bases at positions –1, –2, –3 and –4 in binding, chimeric templates containing a single RNA nucleotide at each of these



**Figure 1.** Sequence and configuration of nucleic acid substrates. (A) Representative sequences of the short DNA (top stand, denoted 'P33' for 'Primer' 33 nt) and long (DNA, or RNA-DNA chimera) strands are shown. Five different short DNA strands that shared a common 5'-end were used. Their lengths were 31, 32, 33, 34 and 35. Only the 33-nt strand that was used for most substrates is shown. In order to illustrate the nomenclature used in the text, the shorter DNA is placed over the complementary bases of three of the several different template strands that were used. The templates were all 50 nt and consisted of either homogeneous DNA or RNA, or chimeras with both DNA and RNA. RNA nucleotides are underlined on the templates. Duplex substrates were named based on the short DNA and template used with the following nomenclature as an example: P33-D18R1(-4)D31. This substrate had the 33 nt DNA hybridized to a template strand where the first 18 5' nt were DNA, followed by a single RNA nucleotide, then 31 DNA nucleotides. The -4 in parentheses indicates the position of the single RNA nucleotide relative to the 3' terminus of P33 with the template nucleotide hybridized to the 3' terminal base being designated as -1. This basic nomenclature was used for all substrates described in the text. (B) A second duplex with a different sequence that was used in experiments is shown. These substrates are designated 'S2' (sequence 2) in the text. Nomenclature is as stated above.



**Figure 2.** Panels for dissociation rate constants ( $k_{off}$ ) (A), and dissociation equilibrium constants ( $K_d$ ) (B). (A) Representative assays are shown to illustrate how dissociation rate constants were determined. This set of assays corresponds to the section in Table 1 labeled 'Binding of RT to duplexes with one or two RNA nucleotides in the template'. Primer labeled with  $^{32}P$  at the 5'-end was used in the assays. The level of primer extension over time was quantified using a phosphoimager as described in 'Materials and Methods' section and these values were plotted to determine  $k_{off}$ . Time points used for the P33-D50 assay were 0, 15 s, 30 s, 1 m, 1.5 m, 2 m, 2.5 m, 3 m and 4 m, while for all other assays time points were 0, 30 s, 1 m, 2 m, 4 m, 8 m and 16 m. Lane A in each panel shows a reaction in the absence of enzyme. Lane B shows a control reaction to test the effectiveness of the heparin trap (see 'Materials and Methods' section). In this reaction, the enzyme was mixed with the trap and the mixture was added to the substrate in the presence of dNTPs and divalent cation and incubated for 10 min before termination. Lane C shows a full extension control in which enzyme was incubated with the substrate as in the trap control reaction except trap was omitted to allow all the bound primer to be extended. (B) Representative assays are shown to illustrate how dissociation equilibrium constants were determined. Panels are labeled at the top with the primer-template that was used in the assay. The concentration of RT (nM) is noted above each lane. Other labels are as above for 2A. Refer to Table 1 for  $K_d$  results.

positions were constructed. All were hybridized to the P33 DNA and  $k_{\text{off}}$  was measured for each. RT dissociated from the substrate with a single RNA base at position  $-3$  (P33-D17R1( $-3$ )D32) at approximately the same rate as the P33-D15R1( $-1$ )D34 template, suggesting that both the  $-1$  and  $-3$  position nucleotides confer a modest increase in binding stability over DNA. In contrast, RT dissociated from the P33-D16R1( $-2$ )D33 substrate  $\sim 3$  times more slowly than the  $-1$  and  $-3$  substrates. Finally, RT dissociated from the P33-D18R1( $-4$ )D31 substrate  $\sim 2$  times more slowly than the  $-2$  substrate, and approximately twice as fast as the substrate containing all four RNA bases (P33-D15R4D31). Since the  $-2$  and  $-4$  positions appeared to be most important to the slow dissociation of RT from RNA–DNA, a template containing both RNA nucleotides (D16R1( $-2$ )D18R1( $-4$ )D31) was constructed and tested. Dissociation of RT from a substrate with this template (P33-D16R1( $-2$ )D18R1( $-4$ )D31) was very slow, such that the off-rate was difficult to measure under the conditions used. It is listed as being at least as slow as the slowest rate measured (that for P33-D15R5D30) on Table 1. Taken together, these results indicate that a single RNA nucleotide at the  $-4$  position results in RT dissociating  $\sim 14$  times more slowly than from a complete DNA strand (D50), while RT binds to templates with both the  $-2$  and  $-4$  RNA nucleotides even more stably than a complete RNA strand [based on D15R5D30 binding even more stably to RT than a complete RNA strand (see above)].

#### **A uridine residue at the $-4$ position promotes more stable HIV-RT binding than other RNA nucleotides**

It was interesting that, in addition to the importance of the  $-4$  RNA nucleotide (P33-D18R1( $-4$ )D31), the  $-2$  position (P33-D16R1( $-2$ )D33) also had a significant effect on RT dissociation. Further, both of these bases were uridines, suggesting that there may be some base preference for promoting stable RT binding. To test this theory, the U at the  $-4$  position was changed to A, G and C with the corresponding base in the P33 DNA also changed to maintain complementarity. Dissociation rates from these substrates were measured, and RT was noted to dissociate more rapidly from all of them. The  $k_{\text{off}}$  was increased  $\sim 3$ -fold when the U was replaced by an A (P33-D18R1( $-4$ U>A)D31) or a G (P33-D18R1( $-4$ U>G)D31) and  $\sim 2$ -fold for a C (P33-D18R1( $-4$ U>C)D31) replacement. In contrast, changing the  $-2$  position from a U to either an A (P33-D16R1( $-2$ U>A)D33) or a C (P33-D16R1( $-2$ U>C)D33) did not significantly change the  $k_{\text{off}}$ . These results indicate that binding to RT is most stable if the single RNA nucleotide in the template is a uridine at the  $-4$  position, though it is also important to note that other RNA residues at this position lead to  $k_{\text{off}}$  values that were several fold lower than a homogenous DNA.

#### **Moving the $-4$ uridine residue to different positions relative to the DNA 3' recessed terminus increases HIV-RT's dissociation rate, confirming the importance of the $-4$ position in the RNA**

The above experiments do not rule out the possibility that the specific sequence context of the  $-4$  nt in this particular substrate, rather than the position relative to the 3' recessed terminus, is the major determinant for slow dissociation. To test this further, the position of the  $-4$  uridine relative to the DNA 3' terminus was shifted using DNAs of various lengths. Oligonucleotides P31, P32, P34 and P35 place the single RNA base in D18R1( $-4$ )D31 at the  $-2$ ,  $-3$ ,  $-5$  and  $-6$  position, respectively, relative to the 3' terminus. These substrates all showed reduced binding to RT as compared to P33-D18R1( $-4$ )D31. Placing the RNA base in the  $-5$  position (P34-D18R1( $-4$ > $-5$ )D31) was least detrimental, resulting in a  $\sim 2$ -fold increase in  $k_{\text{off}}$ , while the  $-2$  (P31-D18R1( $-4$ > $-2$ )D31),  $-3$  (P32-D18R1( $-4$ > $-3$ )D31) and  $-6$  (P35-D18R1( $-4$ > $-6$ )D31) positions increased  $k_{\text{off}}$   $\sim 5$ -, 10- and 6-fold, respectively. The above results illustrate the importance of an RNA nucleotide at the  $-4$  position for stable binding of RT. In addition, they show that moving this position further away from the recessed 3' DNA terminus, as in P34-D18R1( $-4$ > $-5$ )D31, is less unfavorable than moving it nearer.

#### **The 2'-hydroxyl groups on the RNA nucleotides are not required to stabilize binding of HIV-RT**

As was noted in the introduction, several interactions between 2'-hydroxyl groups and RT amino acids have been proposed based on the crystal structure of HIV-RT bound to RNA–DNA (6). Many of these are hydrogen bonds involving the hydroxyl at the 2' position of the RNA nucleotide. To determine if the 2'-hydroxyl was pivotal for stabilizing RT binding on RNA–DNA, modified versions of D15R5D30 and D18R1( $-4$ )D31 in which the RNA nucleotide hydroxyls were converted to 2'-*O*-methyls were tested (P33-D15methylR5D30 and P33-D18methylR1( $-4$ )D31). In both cases, RT  $k_{\text{off}}$  values were similar compared to the templates with 2'-hydroxyl RNA bases (Table 1), indicating that the 2'-hydroxyls do not play a role in stabilizing binding.

#### **Slower dissociation of HIV-RT from RNA–DNA is not dependent upon sequence**

Both the previous work (12) and the work described above were completed utilizing a single template sequence. It was possible, therefore, that some of the findings could be unique to the particular sequence used. To address this concern, a substrate with a different sequence was constructed and analyzed [Figure 1 sequence 2 (S2)]. Although the sequence design was essentially arbitrary, uridine was intentionally excluded from the  $-2$  and  $-4$  positions. By subsequently converting the  $-4$  base to uridine, we could then test whether the conclusions from the previous substrate, which indicated a strong preference for uridine at the  $-4$  position in stable binding to RT, were upheld under these new conditions. Overall, results

indicated that RT dissociated much faster from this new sequence. For instance, a ~10-fold increase in the  $k_{\text{off}}$  value was observed for the complete DNA template compared to the previous sequence (compare S2P33-D50 in Table 1 to P33-D50). Additionally, the five RNA nucleotide version of this template (S2P33-D15R5D30), while dissociating much more slowly from RT than the complete DNA-DNA duplex (93-fold), still showed a ~5-fold higher  $k_{\text{off}}$  than the previous sequence.

A substrate with only one RNA nucleotide at the -4 position (S2P33-D18R1(-4)D31) also showed an ~4-fold increase in  $k_{\text{off}}$  compared to S2P33-D15R5D30, though it still bound much more stably than a complete DNA-DNA duplex (~17-fold). Consistent with a preference for U at the -4 position, converting the -4 position from an adenine to a uridine and the corresponding base in the 33-mer DNA to an A resulted in ~2-fold decrease in  $k_{\text{off}}$  (S2P33-D18R1(-4A>U)D31 compared to S2P33-D18R1(-4)D31). Including RNA bases at both the -2 and the -4 positions resulted in a  $k_{\text{off}}$  value that was essentially the same as the substrate with 5 nt of RNA (compare S2P33-D15R5D30 and S2P33-D16R1(-2)D1R1(-4)D31), just as it did for the first sequence tested. Overall, results with the second template support a role for the -2 and -4 nucleotides, as well as the preference for U over other bases at the -4 position, in promoting stable binding of HIV-RT to RNA-DNA.

While this is the case, they also highlight distinct sequence-dependent differences. This was the case in our previous work as well, where various primers were used to position RT at different locations along a 50-nt template identical to the first template used in this work. Off-rates differed several-fold and were dependent on the position of the primer relative to the template termini as well as the particular sequence to which the primer was bound. Specifically, the difference in off-rates for RNA-DNA versus DNA-DNA duplexes varied from as little as 7-fold to as much as 90-fold more stable binding to the former as compared to the latter (12).

#### **The equilibrium dissociation constant ( $K_d$ ) for HIV-RT is nearly the same for RNA-DNA and DNA-DNA despite large differences in $k_{\text{off}}$**

Since the equilibrium dissociation constant is dependent on the on- and off-rate ( $K_d = k_{\text{off}}/k_{\text{on}}$ ), the lower  $k_{\text{off}}$  values for the RNA-DNA heteroduplex compared to DNA-DNA homoduplex would imply a higher affinity for the former. This would be reflected in a lower  $K_d$  for the heteroduplexes providing that no change in the on-rate had occurred. Assays were performed to measure the  $K_d$  of RT for P33-D50, P33-D15R5D30, P33-D15methylR5D30, P33-D16R1(-2)D33 and P33-D15R1(-1)D34. Surprisingly, RT had similar  $K_d$  values for all five substrates (Table 1 and Figure 2B), even though dissociation was 38 and 30 times faster from P33-D50 than P33-D15R5D30 and P33-D15methylR5D30, respectively, and 8.2 and 2.2 times faster from P33D16R1(-2)D33 and P33-D15R1(-1)D34, respectively. The same phenomenon was observed for AMV-RT with P33-D50 and P33-D15R5D30, both of which yielded similar  $K_d$  values

despite different  $k_{\text{off}}$  values (see below). These results imply changes in  $k_{\text{on}}$  values for the different substrates that are approximately proportional to the changes in  $k_{\text{off}}$  (see 'Discussion' section).

#### **Avian myeloblastosis virus reverse transcriptase and Moloney murine leukemia virus reverse transcriptase show more modest preference for binding to RNA-DNA versus DNA-DNA**

In order to determine if the behavior of HIV-RT is typical for other reverse transcriptases, the binding of AMV- and MuLV-RTs on various substrates was also examined. With regard to P33-D50, both AMV-RT and MuLV-RT bound considerably more stably than HIV-RT (~9- and 6-fold, respectively, Table 1). Like HIV-RT, AMV-RT bound even more stably to P33-D15R5D30 [none of the enzyme used here showed significant RNase H-directed cleavage of the substrate (data not shown)]. Binding was so tight that no consistently measurable dissociation was detected under the conditions used. In contrast, MuLV-RT bound this substrate only ~2-fold more stably than the DNA-DNA. Also consistent with HIV-RT,  $K_d$  values for AMV-RT on P33-D50 and P33-D15R5D30 were similar despite the large  $k_{\text{off}}$  difference.

It is important to note that the D15R5D30 template was optimized for binding to HIV-RT using the HIV-RT E478>Q mutated RT (12). It is possible, therefore, that the 5-nt RNA region may not be ideal for the other enzymes tested here. To further evaluate duplex binding properties, each enzyme was additionally examined on P33-D50 and P33-R50. These experiments were carried out in the absence of  $\text{Mg}^{2+}$  in the dissociation phase in order to prevent cleavage of P33-R50. The KCl concentration was lowered to 20 mM to compensate for the lower binding stability observed in the absence of  $\text{Mg}^{2+}$  (4). Under these conditions, all the enzymes bound more stably to the RNA-DNA duplex, however, HIV-RT bound 27-fold more stably, while the other enzyme showed only ~2-fold better binding to RNA-DNA.

## **DISCUSSION**

### **Slower dissociation of HIV-RT from RNA-DNA versus DNA-DNA does not require 2'-hydroxyls and is dependent on the RNA nucleotides at the -2 and -4 positions**

In this report, we show that HIV-RT's several fold slower dissociation from binary complexes with RNA-DNA versus DNA-DNA requires just two RNA nucleotides (-2 and -4 positions) of the RNA strand and does not require 2'-hydroxyl groups. Similar  $K_d$  values for all duplexes tested (Table 1) suggested that changes in  $k_{\text{on}}$  that were approximately proportional to the changes in  $k_{\text{off}}$  for the various substrates were occurring (see below). The differential stability of the binary complexes could be especially relevant for RT binding to RNA fragments for degradation (see below).

Replacement of 2'-hydroxyl groups on pivotal RNA nucleotides with 2'-O-methyl groups had no effect on

RT's  $k_{\text{off}}$  or  $K_{\text{d}}$  for RNA-DNA hybrids (Table 1). Structural data suggest that the important  $-2$  nt interacts with glutamic acid 89 (E89) of RT by a hydrogen bond to the 2'-hydroxyl group, while an interaction between the pivotal  $-4$  base and isoleucine 94 (I94) is also proposed (6). While the 2'-*O*-methyl modification would have abrogated the E89 interaction to  $-2$ , it is unclear if the I94 interaction would have been affected. There is always the possibility that novel interactions that promote stable binding to RT occur with 2'-*O*-methyl groups. However, the  $k_{\text{off}}$  values were essentially identical for the 2'-*O*-methyl and 2'-hydroxyl versions of the substrates containing the 5-bp RNA-DNA hybrid region (Table 1, compare P33-D15R5D30 and P33-D15methylR5D30) and the substrate with a single RNA at the  $-4$  position (compare P33-D18R1(-4)D31 and P33-D18methylR1(-4)D31). The  $K_{\text{d}}$  values were also the same for methylated and non-methylated versions of the 5-bp RNA-DNA substrates. It is therefore unlikely that novel interactions with 2'-*O*-methyls would have influenced RT binding to the same extent as 2'-hydroxyl interactions. Taken together, these results strongly suggest that amino acid interactions with RNA 2'-hydroxyls do not play a major role in stabilizing the binding of RT to RNA-DNA.

#### The H-form versus B-form structure of RNA-DNA and DNA-DNA hybrids, respectively, is the most likely explanation for HIV-RT's slower dissociation from RNA-DNA

The results from this report provide strong evidence in favor of an alternative explanation for why HIV-RT binds more stably to RNA templates; however, there is no clear indication as to what this alternative is. Some possibilities include (i) the proposed H-form structure of RNA-DNA hybrids is more conducive to stable binding than the B-form structure of DNA-DNA; (2) differences in 'flexibility' between DNA-DNA and RNA-DNA hybrids (19) allow the latter to more easily conform to RT's angled binding cleft; and (3) RT binds stably to the chimeric RNA-DNA substrates tested here not because they 'mimic' RNA-DNA but because they induce a bend or kink in the substrate that is favorable to RT binding. The last possibility was largely discredited in a previous report with chimeric nucleic acids (12). Of the remaining two, we favor the first.

The first possibility would seem to be weakened by the fact that RT's slow dissociation from RNA can be mimicked by duplexes with only short stretches of RNA-DNA. Duplexes with 4- and 5-nt RNA-DNA hybrid regions may take on some H-form qualities over the short stretch, but this seems less likely for the duplexes that had only one or two RNA nucleotides. Still, these individual nucleotides may make it easier for the substrate to transition to a structure that conforms to RT's binding cleft. Pertinent to this was the preference for uridine at the  $-4$  position, which may also make the transition easier. Crystal structures of RT bound to DNA-DNA and RNA-DNA duplexes show that the  $\sim 4$ -bp hybrid region proximal to RT's polymerase active site has a structure similar, but not identical, to A-form nucleic acid (the

structure formed by RNA-RNA hybrids), followed by a  $\sim 40^\circ$  bend that occurs over 4 bp. The remaining duplex in the binding cleft is closer to B-form, similar to normal DNA-DNA (6,7). Interestingly, the A-form portion of the duplex is nearly the same size as the 4-5-bp RNA-DNA hybrid region required for maximal binding stability in these experiments and is positioned in the exact same region as the A-form DNA-DNA or RNA-DNA in the crystals. Although RNA-DNA is H-form rather than A-form, H-form, a 'hybrid' of A- and B-forms, is closer in structure to A-form than B-form is (11). Therefore, RT may have to contort DNA-DNA hybrids more in order to get them to fit properly into the active site, leading to less stable binding.

#### Both on- and off-rates appear to be different for RNA-DNA and DNA-DNA duplexes

Differences in  $k_{\text{off}}$  values between RNA-DNA and DNA-DNA bound to RT in binary complexes were not reflected in  $K_{\text{d}}$  values, which were similar for those duplexes that were tested (Table 1). These findings are consistent with other reports that show similar  $K_{\text{d}}$  values, despite different off-rates, for RT binding to DNA-DNA and RNA-DNA (3,14) and minus strand initiation complexes with tRNA<sub>lys</sub>-RNA versus the same RNA primed with an 18-nt DNA that is homologous to the last 18 3' nt of tRNA<sub>lys</sub> (20). In that case, although  $K_{\text{d}}$  values differed by only  $\sim 2$ -fold, the off-rate of the DNA-primed complex was  $\sim 200$  times slower. The  $K_{\text{d}}$  values reported for both duplex types analyzed here are also similar in magnitude to those reported by others in comparative studies, though the relative difference between  $k_{\text{off}}$  values for RNA-DNA and DNA-DNA are, in general, greater than previously reported (3,14,21). It is possible that this difference could be sequence specific. Likewise, such a difference may also stem from the very tight binding of RT to the chimeric substrate (P33-D15R5D30), which bound RT about  $\sim 2.5$ -fold more stably than the substrate with a complete RNA strand in our previous experiments (12).

Overall the similar  $K_{\text{d}}$ , but vastly different  $k_{\text{off}}$ , values imply large differences in the  $k_{\text{on}}$  for RNA-DNA and DNA-DNA. However, for all the substrates tested in the current report to converge to a similar  $K_{\text{d}}$  value, approximately proportional changes in the on-rates, which compensate for the widely differing off-rates, must be invoked. This suggests that achieving a more stable binding state requires additional time or steps that are reflected in a slower on-rate. Apparently, this also results in an approximately equal decrease in the off-rate. In comparing DNA-primed RNA and DNA templates, Whörl *et al.* (21) noted a slow isomerization step that occurred after initial binding of RT to the substrate. The RNA-DNA substrate showed a greater propensity to undergo the isomerization step and form a 'productive' complex. The authors also suggest, as we do, that structural differences between the duplexes make it easier for RNA-DNA to conform to the RT binding cleft. Since  $K_{\text{d}}$  values are similar for both duplex types, any bound state of RT, including those that have or have not undergone isomerization and those with more or less stable



isomerized states, must quickly convert to a catalytically competent form upon ternary complex formation. This transition occurs without significant dissociation of RT from the substrate, leading to similar  $K_d$  values for each duplex type. This is consistent with biochemical analysis indicating that dNTP binding stabilizes the RT-substrate complex (14,22,23). In turn, the biochemical data are consistent with crystal structures of RT in binary and ternary complexes with DNA–DNA duplexes. Nucleotide binding leads to significant closure of the gap between the finger and thumb domains as they close down on the primer-template and stabilize substrate binding (9).

### Structural differences between HIV-, AMV- and MuLV-RT may explain differences in duplex binding

Results indicated that all tested RTs exhibited a preference for binding RNA–DNA, although clear quantitative differences existed between each. A comparison of RT binding to P33-D50 versus P33-D15R5D30 in the presence of  $Mg^{2+}$  and to P33-D50 versus P33-R50 in its absence revealed that HIV-RT showed the most difference between the different duplexes, followed by AMV-RT, and, finally, by MuLV-RT, which showed the least difference. It is notable that MuLV-RT is the only monomeric enzyme in this group, while HIV- and AMV-RTs are both heterodimers (1). Perhaps even more relevant are the extensive differences observed in crystal structures of MuLV-RT compared to HIV-RT (24,25). The single available crystal structure of the MuLV-RT complete monomer suggests that the duplex trajectory is significantly different compared to HIV-RT as are the positions of the finger and thumb domain. Computer modeling also suggests significant differences in how the template bends while traversing through the binding clefts of the two enzymes (24). Therefore, the structural explanation provided here, which relies on the B to A form transition of the duplex while bound to HIV-RT leading to more stable RNA–DNA binding, may not be relevant for MuLV-RT. A crystal structure of MuLV-RT with a clearly resolved nucleic acid duplex would help to answer these questions.

### Biological relevance for different RT binding states on DNA–DNA and RNA–DNA

The ability of RT to form a stable, tight binding complex on RNA–DNA in the absence of dNTPs may aid in carrying out RNase H cleavage of RNA fragments that remain bound to the template after DNA synthesis. Since RT does not completely degrade the RNA genome during synthesis, secondary cleavage events are required to remove fragments that remain associated with the nascent DNA or to process important regions of the genome, such as the polypurine tract, so it can be used for second strand priming (26–29). The orientation of RT during secondary cleavage places the polymerase domain at the 5' recessed end of the RNA fragment where extension cannot occur and dNTP binding is unlikely (30–33). Therefore, it is important for RT to be able to bind RNA–DNA stably even in the absence of dNTPs.

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