Parameters That Influence the Binding of Human Immunodeficiency Virus Reverse Transcriptase to Nucleic Acid Structures†

Jeffrey J. DeStefano,‡ Robert A. Bambara,‡,§ and Philip J. Fay*,‡,∥

Department of Biochemistry, Department of Medicine, and Cancer Center, University of Rochester, Rochester, New York 14642

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ABSTRACT: We have investigated the binding of human immunodeficiency virus reverse transcriptase (HIV-RT) to various hybrid RNA–DNA or DNA–DNA nucleic acid structures. Binding was measured by preequilibrating the RT with the nucleic acid substrate in the presence or absence of Mg^{2+} and then initiating synthesis or RNase H degradation reactions in the presence of excess "trap" polymer [poly(rA)-oligo(dT)]. The trap polymer sequestered RT molecules as soon as they dissociated from the substrate, such that the amount of synthesis or degradation on the substrate was proportional to the amount of bound RT. On hybrid substrates that had the 3′ terminus of a complementary DNA oligomer recessed on a longer DNA or RNA template, binding to the RNA–DNA hybrid was more stable. Both the dissociation rate constant (k_{off}) and equilibrium constant (K_d) values were larger for the DNA–DNA substrates by 5–10-fold. The difference was clearly in dissociation, since the association rate constant (k_{on}) for both types of substrates was similar. On hybrid structures that had the 3′ termini of a complementary RNA or DNA oligomer recessed on a longer DNA template, k_{off} values are approximately the same on either structure. Although binding of the RT to DNA–DNA hybrid structures did not require Mg^{2+}, its presence during the preequilibration period greatly stabilized binding. An approximate 20–60-fold decrease in the k_{off}, depending on the substrate structure, was observed with Mg^{2+}. Measurements on one particular DNA–DNA hybrid indicated that the k_{on} decreased by approximately 2 orders of magnitude with Mg^{2+}. The relevance of these results to HIV replication is discussed.

Retroviral replication requires the conversion of a single-strand RNA genome to double-stranded DNA (Varmus & Swanstrom, 1984). The reverse transcriptase (RT)[1] is the key enzyme involved in this conversion. It is a multifunctional enzyme possessing DNA- and RNA-dependent DNA polymerase activity and RNase H activity [diMarzo *et al.*, 1986; Hansen *et al.*, 1987; Starnes & Cheng, 1989; Varmus & Swanstrom, 1984; for a review, see Goff (1990)]. An activity that can cleave RNA in an RNA–RNA duplex has also been detected (Ben-Artzi *et al.*, 1992).

In the course of duplex DNA synthesis, a variety of different nucleic acid structures are generated. The most widely accepted model for retroviral replication suggests that RNA–RNA, RNA–DNA, and DNA–DNA hybrid structures, as well as single-stranded RNA and DNA, are simultaneously present (Varmus & Swanstrom, 1984). It is likely that the RT would display an order of preference for binding the various structures. Determination of the binding affinities of the RT for these different structures, and the stabilities of the RT–polynucleotide complexes, could provide insight into control of the order of steps in synthesis of the viral chromosome. For example, the RT could bind more stably to hybrid structures that are likely to be present during first-strand, compared to second-strand, DNA synthesis. This would mean a preference for binding RNA–DNA hybrids in which RNA is the template strand. There might be a lesser preference for RNA oligomers (partially degraded genomic RNA) bound to nascent DNA present after first-strand synthesis, or the DNA–DNA hybrids of second-strand synthesis. The relative affinity of the RT for these structures may be important in assuring that the majority of first-strand synthesis is completed before second-strand synthesis, which uses the newly synthesized first strand of DNA as template, is emphasized.

It has been shown in our laboratory (DeStefano *et al.*, 1992) and by others (Yu & Goodman, 1992) that HIV-RT generally binds with greater stability, as indicated by the dissociation rate constant, to DNA oligonucleotide-primed RNA substrates (RNA–DNA) in comparison to DNA substrates (DNA–DNA). In this paper, we have investigated the binding of HIV-RT to hybrid structures in more detail. We have determined what reaction parameters and what structural features of duplex substrates influence binding. Our results demonstrate that the RT binds with greater stability to DNA oligonucleotide-primed RNA templates than to DNA templates. It also binds with higher affinity, as indicated by the dissociation equilibrium constant. However, when a DNA template strand is hybridized to an RNA or a DNA oligomer, such that the RNA or DNA oligomer could potentially serve as a primer for DNA synthesis, the RT–RNA–DNA or DNA–DNA complexes have about equal stability. In addition, we found that RT binding to DNA–DNA duplexes did not require, but was greatly stabilized by, Mg^{2+}.

MATERIALS AND METHODS

Materials

Recombinant HIV-RT, having native primary structure, was graciously provided to us by the Genetics Institute (Cambridge, MA). This enzyme had a specific activity of

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* Address correspondence to this author at the Hematology Unit, P.O. Box 610, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-6576. Fax: (716) 473-4314.

‡ Department of Biochemistry.

§ Cancer Center.

∥ Department of Medicine.

[1] Abbreviations: RT, reverse transcriptase; RNase H, ribonuclease H; HIV, human immunodeficiency virus.

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approximately 40 000 units/mg. One unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37 °C using poly(rA)-oligo(dT) as template-primer. Aliquots of HIV-RT were stored frozen at −70 °C, and a fresh aliquot was used for each experiment. T4 polynucleotide kinase and T7 RNA polymerase were obtained from United States Biochemical Corp. Placental RNase inhibitor, rNTPs, and all restriction enzymes were obtained from Boehringer Mannheim Biochemicals; dNTPs and Sephadex G-50 were obtained from Pharmacia. Poly(rA) and oligo(dT)$_{16}$ were obtained from Midland Certified Reagent Co. The DNA oligonucleotides used as primer strand (see Figure 1) were synthesized by Genosys Inc. (Houston, TX). All other chemicals were from Sigma Chemical Co. Radiolabeled compounds were from New England Nuclear or Amersham.

Methods

Determination of Dissociation Equilibrium Constants (K_d) by Nucleotide Incorporation. HIV-RT (final concentration 50 nM for DNA–DNA substrates or 6.7 nM for RNA–DNA substrates) was preequilibrated with substrate (1–5 nM for 30R25D and 30R30D or 2–32 nM for 30D25D) for 10 min at 37 °C in 15 μL of 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 2% glycerol (w/w), 0.2 mM EDTA, and 5 mM KCl (buffer A). Assays were initiated by the addition of $MgCl_2$, 5 μg of poly(rA)–oligo(dT)$_{16}$, and the next template-directed dNTP (specific activity 400 Ci/mmol) to be added to the primer strand, in 10 μL of buffer A to give a final concentration of 6 mM $MgCl_2$ and 1 μM dNTP. The poly(rA)–oligo(dT)$_{16}$ was used as a trap to bind and sequester RT molecules that had dissociated from substrates (DeStefano *et al.*, 1991). In some reactions, $MgCl_2$ (6 mM) was included during the preequilibration period. Reactions were run for 15 s at 37 °C and were terminated by the addition of 25 μL of 50 mM EDTA. Unincorporated nucleotides were removed by centrifugation through 0.5-mL G-50 spin-columns (Penefsky, 1977). The spun samples were dried in a Speed-Vac (Savant) and resuspended in 20 μL of gel electrophoresis loading buffer [45% formamide (v/v), 5 mM EDTA (pH 8.0), 0.05% (w/v) xylene cyanol, and bromophenol blue]. Samples were loaded onto a 12% polyacrylamide/7 M urea sequencing gel and subjected to electrophoresis as described below. The gels were covered with Saran Wrap and exposed at −70 °C. Bands corresponding to primer strands that had incorporated radiolabeled dNTPs were excised, mixed with scintillation fluid, and counted. The level of RT-bound substrate was determined on the basis of incorporation, and Scatchard plots were constructed to calculate the K_d values and the concentration of active enzyme in the assays. Poly(rA)–oligo(dT)$_{16}$ used in the reactions was prepared by mixing oligo(dT)$_{16}$ with poly(rA) at a 1:8 ratio (w/w) in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The mixture was incubated for 30 min at 37 °C and then cooled slowly to room temperature.

Determination of Dissociation Rate Constants (k_{off}) by Nucleotide Incorporation. Dissociation rates were determined by a modification of the assay to determine K_d values. Reaction conditions and the preequilibration period were the same, and the substrate concentration was 4 nM. After preequilibration, 5 μg of poly(rA)–oligo(dT)$_{16}$ in 5 μL of buffer A was added to the reactions, and incubation was continued for 0–80 s in some experiments, or for 0–320 s in others as indicated on the figures. Reactions were then initiated with $MgCl_2$ and dNTPs in a volume of 5 μL as described above. In some assays, Mg^{2+} (6 mM) was included during the preequilibration period and with the poly(rA)–oligo(dT)$_{16}$. Samples were treated as described above. The dissociation rates were determined by constructing a graph of incorporation, relative to the time zero sample, *vs* time. A least-squares fit of the data to an equation for single-exponential decay [$f(x) = ae^{-bx}$, where $a = 1$ and b is the dissociation rate] using the Macintosh Sigma Plot program (Jandel Corp.) was used to construct the graphs and determine the k_{off}.

K_d and k_{off} Values As Determined by RNase H Activity. The conditions for these assays were as described above except that only Mg^{2+} was added to initiate the reactions. Reactions were terminated by the addition of 2 times gel loading buffer, and then loaded directly onto sequencing gels for electrophoresis. In these reactions, the RNA portion of the RNA–DNA hybrid was labeled internally as described below under Runoff Transcription. For K_d determinations, radiolabeled cleavage products (representing bound substrate) and uncleaved substrate (free substrate) were excised from the gel after electrophoresis, and quantitated as described above. The k_{off} values were determined as described above, except that relative cleavage *vs* time was used to construct the graphs.

Competition Assay for Determining Relative Affinity. The conditions for this assay were the same as for the k_{off} determinations [substrate 30R25D (internally labeled; see Figure 1) was the substrate competed against in these assays] by the RNase H cleavage method with the following exceptions: (1) The concentration of HIV-RT was 3.4 nM. At this concentration, approximately 15% of the added 30R25D was cleaved in the absence of competitor. (2) Competitor substrate was included during the preequilibration period at concentrations ranging from 0 (no competitor) to 8-fold over the competed substrate which was present at 4 nM. The amount of the competed substrate that was cleaved was determined by excision of the cleaved substrate after gel electrophoresis, as described above.

Isolation of Substrates and Preparation and Quantitation of Hybrids. DNA template strands (the longer strand) for substrates 75D25D, 66D30D, and 72D30R or 72D30D (see Figure 1) were isolated from pBSM13+ by cleavage with restriction endonucleases. For the template strand of substrates 75D25D and 72D30R or 72D30D, the plasmid was cleaved with *Hae*III and *Pst*I, while for substrate 66D30D, *Mse*I and *Sac*I were used. Samples were subjected to electrophoresis on 8% sequencing gels, and single-stranded DNA bands were located by ultraviolet shadowing. Bands 66, 72, and 75 nucleotides in length were excised from the gels. DNA was eluted from the gel and isolated as described under Runoff Transcription. The RNA template strand used for substrate 83R30D was prepared by runoff transcription as described below. Hybrids were prepared by mixing primer and template strands at an approximately 2:1 ratio of 3′ termini, respectively, in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 80 mM KCl. The mixture was heated to 65 °C for 10 min and then cooled slowly to room temperature. To separate hybrids from unhybridized material, the samples were mixed with 6 times concentrated native gel electrophoresis buffer [40% (w/v) sucrose, 0.25% (w/v) xylene cyanol, and bromophenol blue] and loaded onto 12% native polyacrylamide gels. Gels were prepared and subjected to electrophoresis as described (Sambrook *et al.*, 1989). Hybrids were located either by ultraviolet shadowing or by autoradiography and excised and isolated in the same manner as were the transcripts and template strands. Greater than 95% of the isolated material remained hybridized as determined by reelectrophoresis of the samples on a native gel (data not shown). The hybrids were quantitated on the basis of the specific activity

for RNA–DNA hybrids in which the RNA strand was internally labeled. To quantitate DNA–DNA hybrids, the hybrid was incubated, under the same conditions as for the K_d determination, with excess HIV-RT in the presence of the labeled, next template-directed nucleotide to be added to the primer strand. To prevent read-through synthesis caused by misincorporation, the reactions also included a dideoxynucleotide (final concentration 1 μM) which was complementary to the nucleotide following the labeled one. Samples were subjected to electrophoresis, excised, and counted as described above. The level of substrate was determined based on the specific activity of the incorporated nucleotide.

Runoff Transcription. This procedure was performed as described in the Promega Protocols and Applications Guide (1989). Plasmid pBSM13+ was isolated from transformed *Escherichia coli* XL1 Blue cells using a Qiagen plasmid kit as described by the manufacturer. The plasmid DNA was cleaved with various restriction enzymes [*Bam*HI for template strand 30R and *Mse*I for 83R (see Figure 1)]. T7 RNA polymerase was used to prepare runoff RNA transcripts 30 and 83 nucleotides in length for substrates 30R25D or 30R30D, and 83R30D, respectively. For preparation of internally labeled transcripts, 500 μM each of ATP, GTP, and UTP and 50 μM [α-^{32}P]CTP (approximately 10 Ci/mmol) were used in the transcription reactions. The DNA template was digested with bovine pancreatic RNase-free DNase I (1 unit/mg of DNA). The RNA transcript was then extracted with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then ethanol-precipitated twice using 3 volumes of ethanol and a final concentration of 2 M ammonium acetate. The runoff transcript was resuspended and purified by electrophoresis on polyacrylamide gels containing 7 M urea. The full-length transcript was located by autoradiography, excised from the gel, and eluted overnight in a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. The eluate was separated from the polyacrylamide by centrifugation in a microfuge, and subsequent filtration through a 0.45-μm (pore size), 25-mm disposable spyringe filter (Nalgene). The filtrate was then ethanol-precipitated with 3 volumes of ethanol and 0.1 volume of 2 M NaCl.

Gel Electrophoresis. Denaturing polyacrylamide sequencing gels [19:1 acrylamide:bis(acrylamide) ratio], containing 7 M urea, were prepared and subjected to electrophoresis as described (Sambrook et al., 1989).

RESULTS

Design of the Nucleic Acid Structures and Binding Assays Used To Measure HIV-RT Binding. HIV-RT binding was measured on the nucleic acid substrates shown in Figure 1. The substrates were made from DNA restriction endonuclease cleavage products, or transcripts from the plasmid pBSM13+, and synthetic DNA oligonucleotides produced by chemical synthesis. The substrates are named as follows: The first number indicates the length of the template strand (the longer of the two strands in all cases except 30R30D). The first letter indicates whether the template strand was RNA or DNA (R or D). The number that follows indicates the primer strand length. The final letter indicates whether the primer was RNA or DNA. Substrates 30D25D and 30R25D, and 66D30D and 83R30D were used to analyze the binding of HIV-RT to DNA oligonucleotide-primed RNA *vs* DNA substrates. Substrate 75D25D (compared with substrate 30D25D) was used to determine whether the length of the template strand affected RT binding. Substrate 30R30D was used to determine whether the presence of a recessed 3′ DNA terminus (compared to 30R25D) affected binding. Substrates

FIGURE 1: Substrates for binding experiments. Shown is the nucleotide sequence of the template strands used in these experiments. The bracketed area above each template indicates the region where the primer strand hybridized. The name of each substrate is given in parentheses. Refer to Results for an explanation of the nomenclature.

72D30D and 72D30R were used to evaluate the binding of RT to a DNA template hybridized to either an RNA or a DNA oligomer.

Most dissociation rate constants (k_{off}) and dissociation equilibrium constants (K_d) were determined on the basis of the RT-catalyzed, template-directed incorporation of a labeled nucleotide following the 3′-terminal nucleotide on the primer strand. Experiments were performed in the presence of excess trap polymer that effectively sequestered RT molecules that dissociated from the experimental substrate during the time course of the reactions. Since the dissociation of the enzyme–nucleic acid substrate complexes is slow relative to the rate of polymerization (Reardon, 1992), the amount of synthesis was proportional to the amount of bound RT at the initiation of reactions. The primer was extended by one nucleotide, or up to two in the case of substrates 66D30D and 83R30D. On some substrates (30R25D, 30R30D, and 72D30R), binding parameter values were determined by cleavage of the RNA portion of the RNA–DNA hybrid by the RNase H activity of the RT. This assay was performed such that the amount of cleavage is proportional to the amount of bound RT. With substrate 30R25D, the k_{off} values as determined by incorporation of RNase H were similar although not identical (see Table I). The reason for the small variation is not known.

Binding of HIV-RT to DNA Oligonucleotide-Primed RNA vs DNA Substrates. Results from typical k_{off} measurements are shown in Figure 2 (panels A and B, 30D25D and 30R25D, respectively). In the experiment shown in Figure 2A, the RT was preequilibrated, in the absence of Mg^{2+}, with substrate 30D25D for 10 min as described under Methods. The poly(rA)–oligo(dT)$_{16}$ trap was then added. The reactions were initiated by addition of Mg^{2+} and 1 μM [α-^{32}P]dCTP, the first template-directed nucleotide to be added to the primer strand, at different times after trap addition. Values of k_{off} were obtained by fitting the data to an equation for single-exponential decay. Increasing the concentration of dCTP to 5 or 10 μM did not significantly affect the level of dCTP incorporation (data not shown). Figure 2B shows an experiment in which the k_{off} was evaluated based on cleavage of the RNA portion of an RNA–DNA hybrid. For these experi-

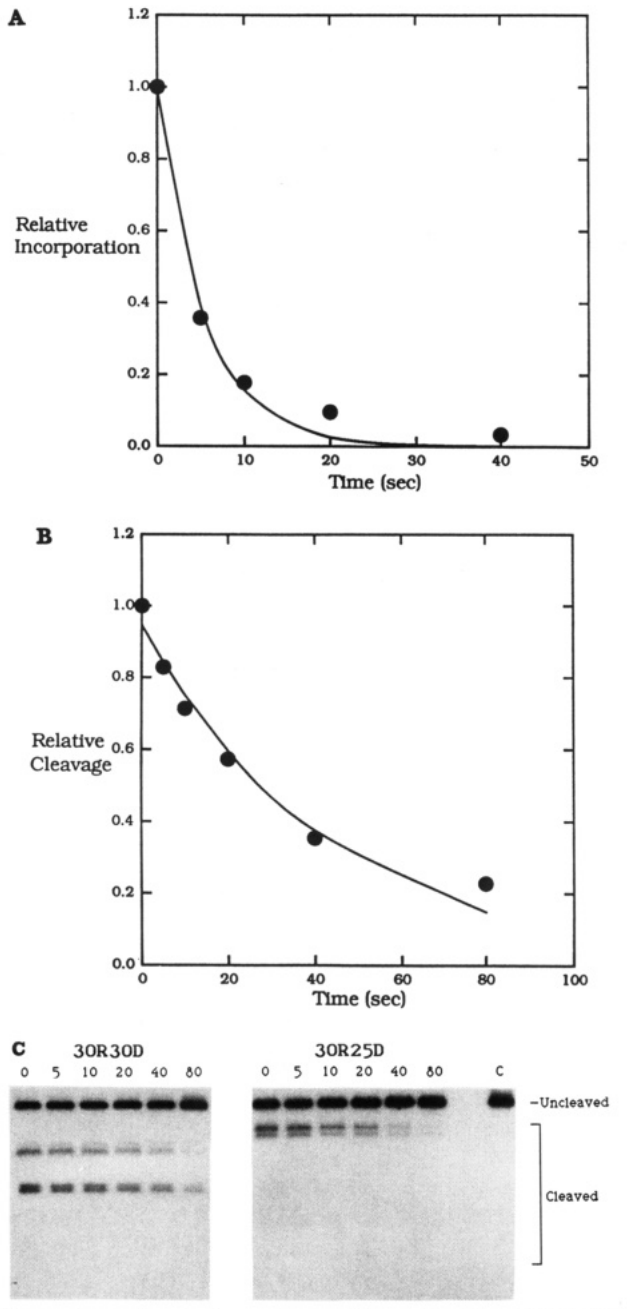

FIGURE 2: Determination of dissociation rates. Dissociation rates were determined by nucleotide incorporation (A) or RNase H degradation (B) as described under Methods. Panels A and B show typical dissociation curves for templates 30D25D and 30R25D, respectively. The curves represent nonlinear least-squares fits of the data from single experiments to an equation for single-exponential decay. Panel C shows autoradiograms of typical dissociation rate experiments (performed by the RNase H cleavage method) for templates 30R30D and 30R25D. The numbers above each lane indicate the time after trap when the reactions were initiated by the addition of Mg^{2+}. Lane C (control) shows uncleaved 30R25D. Positions of uncleaved RNA and RNA cleavage products are indicated.

ments, the dNTP was not included in the initiation mixture, and the RNA was internally radiolabeled.

In Figure 2C, an autoradiogram of a typical experiment used to determine the dissociation rate of HIV-RT from substrates 30R25D and 30R30D is shown. Note that these substrates yielded different cleavage products upon HIV-RT-mediated cleavage. Using 5′-end-labeled 30R RNA, we determined the length of the 5′-derived cleavage products produced by HIV-RT-mediated cleavage of substrates 30R25D and 30R30D. Our results indicated that the products ranged from 24 to 26 and from 19 to 21 nucleotides in length for 30R25D and 30R30D, respectively (data not shown).

On some substrates, a low level of read-through synthesis was detectable. For this to occur, the RT must misincorporate at least one nucleotide. The result was not surprising given the relatively high level of misincorporation reported for this enzyme (Bebenek *et al.*, 1989; Boyer *et al.*, 1992; Ji & Loeb, 1992; Hübner *et al.*, 1992; Preston *et al.*, 1988; Roberts *et al.*, 1988; Takeuhiro *et al.*, 1988; Weber *et al.*, 1989; Yu & Goodman, 1992). In many cases, a portion of the oligo$(dT)_{16}$ was also extended and labeled by one nucleotide. The extension occurred despite the absence of dTTP in all assays. Such an observation is also consistent with the high level of misincorporation by HIV-RT.

For substrates 30D25D, 30R25D, and 30R30D, the K_d value was determined by Scatchard analysis. Representative plots are shown in Figure 3. Plots for 30D25D were linear over the range of substrate concentrations used (Figure 3A). In contrast, for 30R25D (Figure 3B) and 30R30D, plots were curvilinear. With low concentrations of substrate, the bound:free ratio changed very little as the concentration of substrate was increased. This was followed by a steep and linear decrease in the ratio at higher concentrations of substrate. The bound:free ratio at the lower concentrations of substrate may be the practical maximum that can be measured in the experiment. This effect may have occurred because of the relative concentrations of substrate and active RT. The concentration of active RT was about 2.5 nM, as estimated from the x intercept of the graph shown in Figure 3B. This concentration is substantially higher than both the concentration of substrate and the K_d value. Normally, K_d determinations for protein–polymer binding are performed at concentrations of protein below the K_d value. A high concentration of enzyme was used in these assays because at lower concentrations, the amount of cleaved substrate was so low that it approached the detection limits of the assay. The linear portion of the plot was used to compute the K_d value. We note that the points in the linear segments were very consistent with the projected line (regression coefficients of approximately 0.95).

The quantity of RT binding to DNA–DNA substrates, based on incorporation, was much lower than to RNA–DNA substrates. This is reflected in the higher K_d for the binding to DNA–DNA substrates compared to RNA–DNA substrates (Table I). The low binding level was especially evident in the case of 30D25D *vs* 30R25D. Even with the comparatively high quantity of enzyme used, the proportion of substrate bound in assays with 30D25D was much lower than with 30R25D (Figure 3).

The k_{off} and K_d values for the substrates used to evaluate the binding of HIV-RT to DNA oligonucleotide-primed RNA *vs* DNA template strands are shown in Table I. The k_{off} for the RT complex with 30D25D was approximately 5-fold higher than for the complex with 30R25D. The k_{off} values with substrates 66D30D and 83R30D also indicated that the RT remained bound longer when RNA was the template strand. In the latter case, there was about a 10-fold increase in the k_{off} when DNA was the template strand (66D30D). We note that although the DNA oligonucleotides used to prime substrates 66D30D and 83R30D were identical, the DNA and RNA template strands were not completely homologous. The 66D30D template had a 5′ extension of 29 nucleotides out from the 3′ end of the terminus of the bound primer. There was an additional 15 and 2 nucleotides on the 5′ and 3′ ends, respectively, of the RNA template strand (83R). However, since the templates were homologous in the region where the RT would be expected to bind, we do not believe

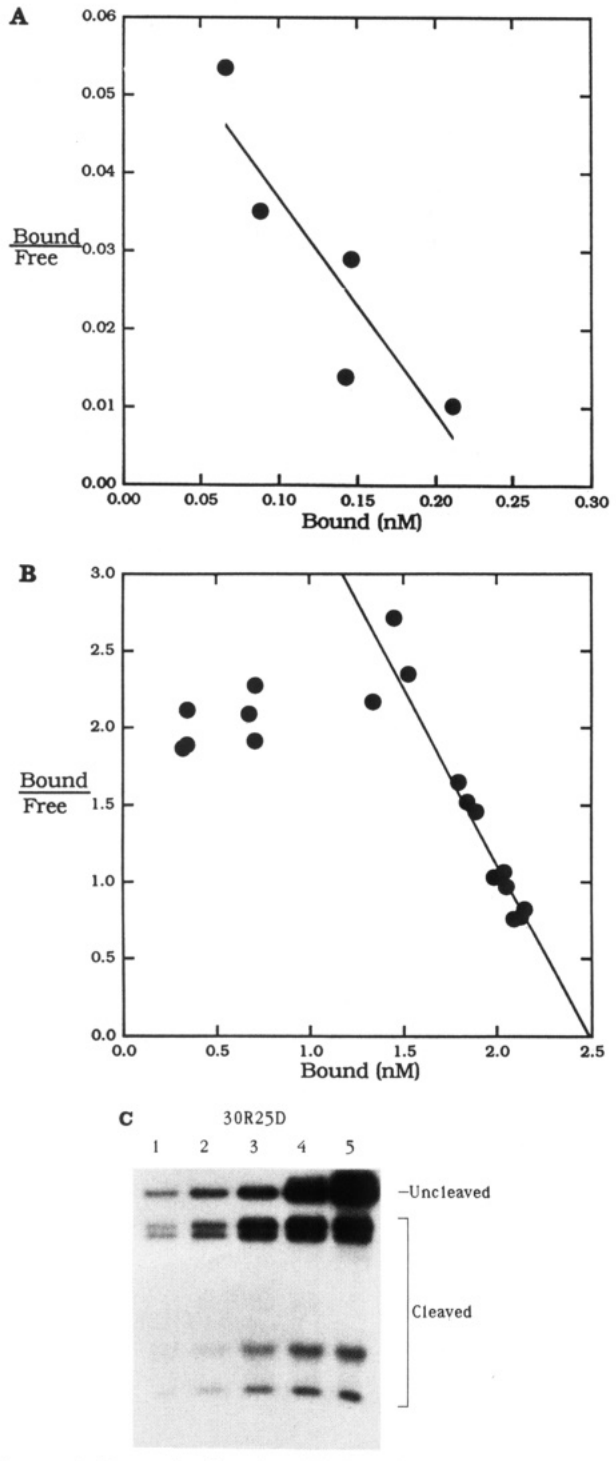

FIGURE 3: Determination of equilibrium dissociation constants. K_d values were determined by nucleotide incorporation (A) or RNase H degradation (B and C) as described under Methods. Panels A and B show typical Scatchard plots for templates 30D25D and 30R25D, respectively. K_d values were determined from the slope of the plots. For panel A, the line represents a linear least-squares fit of the data from a single experiment. Three independent experiments were performed to determine the K_d value for template 30D25D. Regression coefficients were 0.88 or greater. For panel B, the line represents a linear least-squares fit (regression coefficient = 0.95) of the data from a single experiment with individual samples prepared in triplicate. Only data points with values greater than 1 on the x axis were used to construct the line (see text). The slope of the line was used to calculate the K_d. Panel C shows an autoradiogram for a typical experiment with template 30R25D. The positions of uncleaved RNA and RNA cleavage products are indicated.

that the small percentage differences in the template strand lengths were likely to have had a significant impact on the binding results.

Table I: Dissociation Rate Constants (k_{off}) and Equilibrium Dissociation Constants (K_d) of Substrates

substrate	k_{off} (s^{-1})[c]	K_d (nM)
30D25D (I)[a]	0.15 ± 0.03	3.8 ± 0.2[d]
30D25D + Mg^{2+} [b]	0.0027 ± 0.002	9.33 ± 0.09[d]
30R25R (I)	0.023 ± 0.002	
30R25D (H)	0.030 ± 0.007	0.44[e]
30R30D (H)	0.014 ± 0.001	0.52[e]
75D25D (I)	0.026 ± 0.002	
66D30D (I)	0.024 ± 0.003	
83R30D (I)	0.0029 ± 0.0006	
72D30D (I)	0.034 ± 0.003	
72D30D + Mg^{2+}	0.0016 ± 0.0002	
72D30R (H)	0.027 ± 0.002	

[a] "I" indicates determinations were made by the incorporation method while "H" indicates the RNase H method. [b] +Mg^{2+} indicates that $MgCl_2$ (6 mM) was included during the preequilibrium period and the incorporation method was used for determinations. [c] Data from 2–4 independent experiments ± a standard deviation. [d] Data from 3 independent experiments ± a standard deviation. [e] Data from a single experiment, samples prepared in triplicate.

Binding of the RT to substrate 30D25D was particularly unstable. To determine whether the very short template extension (five nucleotides out from the primer terminus) contributed to this instability, we constructed a substrate primed with the same primer as 30D25D but with a much longer template extension (32 nucleotides). The k_{off} for the RT bound to this substrate (75D25D) was about 6-fold lower than that of the RT–30D25D complex (Table I and Figure 2C). This indicated that the longer template strand did improve the stability of the RT–substrate complex for a DNA–DNA substrate.

Curiously, increasing the length of the DNA strand on substrate 30R30D did not have a stabilizing affect on the complex. [Compare the k_{off} for 30R30D with that for substrate 72D30R (Table I). In this case, 30R is the same nucleic acid segment.] This substrate (30R30D) was an RNA–DNA blunt-ended complete duplex. It was designed to determine whether the presence of a recessed 3′ DNA terminus on the RNA template strand was influencing RT binding parameters. Possibly the blunt end is a special case in which unique protein–DNA contacts are made that increase stability.

Evaluation of the K_d for substrates 30D25D and 30R25D indicated about a 8-fold decrease in the value when the template strand was RNA (Table I). Using the K_d and k_{off} values, association rate constants (k_{on}) of 4.17×10^7 and 6.82×10^7 M^{-1} s^{-1} for 30D25D and 30R25D, respectively, were calculated. Thus, the lower K_d value obtained for the RNA template–DNA primer substrate was primarily determined by the lower k_{off}.

Also shown in Table I are the K_d values for binding of the RT to substrates 30R25D and 30R30D. These are approximately the same. Evidently the unique complex stability of binding to a blunt end is not manifest in a higher affinity of binding, because both the association and dissociation rates have been slowed correspondingly.

All experiments shown in Table I were performed using 5 mM KCl in the assays. Higher concentrations of salt destabilize the RT–substrate complexes. For example, with substrate 30D25D, the k_{off} was approximately 5-fold higher when 50 mM KCl was used as compared to 5 mM (data not shown). Note that Mg^{2+} was included during the preequilibration period for some of the determinations on DNA–DNA substrates shown in Table I (as indicated). With the methods used in these experiments, it was not possible to evaluate the effects of Mg^{2+} on substrates containing RNA. This is because they would be degraded by the RNase H activity of the RT during preequilibration.

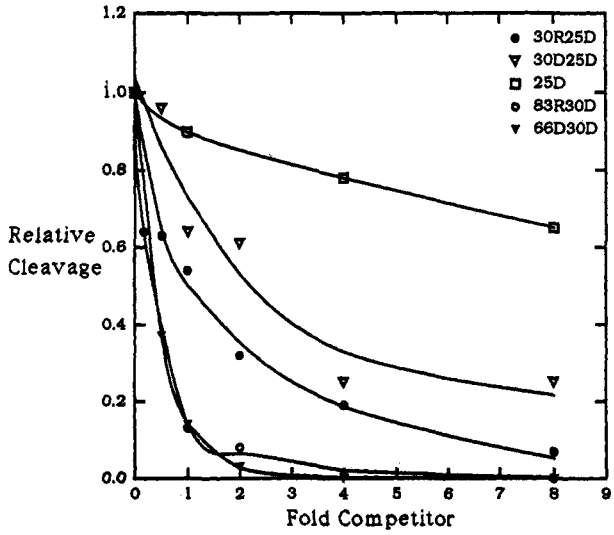

FIGURE 4: Direct competition assay comparing RT affinity for 30R25D to other substrates. The experiment was performed as described under Methods. The substrates used as competitors are indicated on the graph. The curves shown were generated by a nonlinear least-squares fit of the data.

Binding of RT to RNA- or DNA-Primed DNA Substrates. During, or just after, first-strand DNA synthesis, it is likely that nucleic acid structures with relatively small RNA oligomers bound to newly made DNA are present. Results from our laboratory indicate that the RT that is carrying out synthesis on an RNA template only partially degrades that template as it passes over (DeStefano *et al.*, 1991). Therefore, either the leading RT must return at a later time to complete degradation, or other RTs following behind the synthesizing RT must perform that function. Partial degradation by the synthesizing RT may generate nucleic acid structures with a similar configuration to the substrate 72D30R. Such structures may also represent a block to second-strand synthesis. Consequently, we felt that it was important to measure binding to the RT to these complexes. Results showed that the stability of the HIV-RT complex with template 72D was approximately the same whether the primer strand was DNA (72D30D) or RNA (72D30R, Table I). Dissociation rate constants were also similar to those obtained for substrates 75D25D and 66D30D.

Affinity of HIV-RT for Substrates As Evaluated by Direct Competition. In order to directly determine the relative affinity of the RT for different substrates, we performed a series of competition assays (Figure 4). In this assay, a limiting amount of enzyme, relative to substrate, was preequilibrated with a fixed concentration of 30R25D. Also included during the preequilibration was a competitor substrate ranging in concentration from 0 (no competitor) to 8-fold relative to the concentration of 30R25D. As expected, when an equimolar amount of cold 30R25D was included during the preequilibration approximately 50% of the RTs were competed off of the labeled substrate. Substrate 30D25D was a poorer competitor than 30R25D, indicating that 30D25D has a relatively lower affinity for the enzyme. However, note that in the competition assay, only a 2–3-fold excess of 30D25D was required to compete approximately 50% of the RT off of 30R25D. This result suggests an approximate 2–3-fold higher K_d for 30D25D relative to 30R25D. The values in Table I indicate about an 8-fold difference. The reason for the discrepancy is unclear although both methods clearly indicate that HIV-RT binds with greater affinity to 30R25D than to 30D25D. Single-stranded DNA was the least efficient competitor. Substrates 83R30D and 66D30D were also tested.

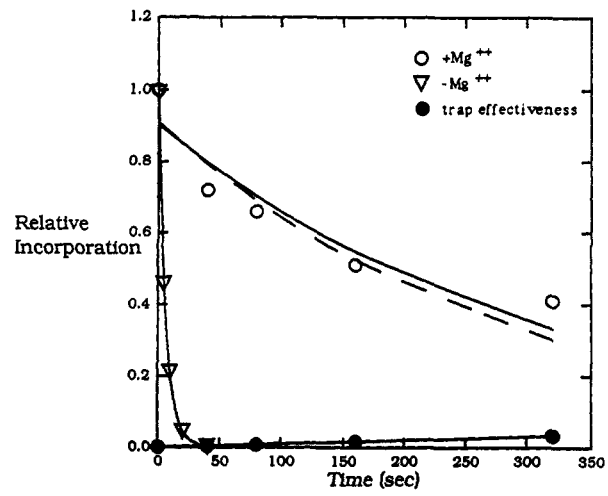

FIGURE 5: Comparison of the dissociation of RT from substrate 30D25D in the presence or absence of Mg^{2+}. Dissociation curves were constructed as described in the legend to Figure 2. Figure 4 shows the dissociation of RT from 30D25D when Mg^{2+} was included in ($+Mg^{2+}$) or omitted from ($-Mg^{2+}$) the preequilibration mixture. Synthesis resulting from rebinding to the experimental substrate in the presence of trap polymer was estimated by incubating the RT, in the presence of [α-^{32}P]dCTP and Mg^{2+}, with trap polymer and experimental substrate for times ranging from 20 to 320 s. This allowed assessment of trap effectiveness. The total incorporation relative to the time zero point for 30D25D, in the presence of Mg^{2+}, was used to construct the curve. The dissociation rate for 30D25D in the presence of Mg^{2+} was calculated after subtracting from the original curve the incorporation that potentially resulted from rebinding (dashed line).

Both had significantly greater affinities for the RT than did 30R25D. However, because these substrates are considerably larger than 30R25D, it is difficult to make direct comparisons between them and 30R25D in this type of assay. The results do indicate that the RT has a significantly greater affinity, on a per mole of full-length substrate basis, for the larger substrates.

Effect of Mg^{2+} on the k_{off} and K_d Values for HIV-RT–Substrate Complexes. Previous work has indicated that the binding of RT to nucleic acid substrates does not require Mg^{2+}. As mentioned above, most experiments were performed in the absence of Mg^{2+} to prevent RNase H-directed cleavage of RNA substrates. It is possible, however, that Mg^{2+} may affect the stability of the RT–substrate complex. To test this hypothesis, substrates 30D25D and 72D30D were preequilibrated with RT in the presence or absence of Mg^{2+}. The preformed complexes were allowed to dissociate in the presence or absence of Mg^{2+}. Results indicated about a 60- and a 20-fold stabilization of preformed complexes for substrates 30D25D and 72D30D, respectively, when Mg^{2+} was included during the preequilibration and dissociation periods (compare 30D25D and 72D30D with or without Mg^{2+}, Table I). Figure 5 shows a graphical comparison of the dissociation curves for substrate 30D25D in the presence or absence of Mg^{2+}. The k_{off} values for the RT–30D25D and RT–72D30D complexes, in the presence of Mg^{2+}, were calculated after taking into consideration the incorporation which potentially resulted from the ineffectiveness of the trap. As can be seen in Figure 4, this had only a small effect on the results of the determination.

The effectiveness of the trap polymer in sequestering the RT is also shown in Figure 5. The trap was essentially 100% effective for time points below about 80 s. In most cases (except when Mg^{2+} was included in the preequilibration, and for measurements with substrate 83R30D), time points of 80 s or less were sufficient to determine k_{off} values. Thus, rebinding of the experimental substrate, in the presence of trap, was not a factor. When longer times are used, the

potential effect from rebinding was taken into consideration when calculating the k_{off}. In all cases, it had only a small effect on the determinations (see Figure 5).

The K_d value for the binding of the RT to substrate 30D25D in the presence of Mg^{2+} was also determined (Table I). Despite the slower dissociation, the K_d value with Mg^{2+} was approximately 3-fold higher than in the absence of Mg^{2+}. This result indicates that Mg^{2+} was slowing association even slightly more than dissociation. From the K_d and dissociation rates for substrate 30D25D in the presence of Mg^{2+}, a k_{on} of 2.89×10^5 M^{-1} s^{-1} was calculated. This value was about 150-fold lower than in the absence of Mg^{2+}.

It was also interesting that the quantity of active enzyme as determined from the x-axis intercept of the Scatchard plots (with 30D25D as substrate) was higher in the presence of Mg^{2+}. There was about a 10-fold difference, 0.26 ± 0.03 and 2.1 ± 0.1 nM, in the absence and presence of Mg^{2+}, respectively, in the estimated concentration of active enzyme. One possibility is that Mg^{2+} stabilizes the form of the RT that can bind duplex nucleic acids, perhaps by stabilizing the heterodimer. In both cases, the concentration was substantially lower than the 50 nM of enzyme added to the reaction as estimated from the molecular weight of HIV-RT. However, the discrepancy was not as great for 30R25D. The estimated level of active enzyme was 2.5 nM from the Scatchard plot for this substrate (Figure 3B), whereas the concentration of RT in the reactions, on the basis of molecular weight, was 6.7 nM. This last result suggests that a greater proportion of the added enzyme is capable of binding to RNA–DNA *vs* DNA–DNA duplexes.

DISCUSSION

We have examined the binding of HIV-RT to duplex nucleic acid substrate structures. Our results indicate that the stability of RT–substrate complexes is greater when the DNA oligonucleotide-primed template strand is RNA rather than DNA (Table I). This conclusion is consistent with previous work in our laboratory (DeStefano *et al.*, 1992) and from others (Yu & Goodman, 1992). Results with substrates 30D25D and 30R25D also showed that the RT has greater affinity for DNA oligonucleotide-primed RNA *vs* DNA substrates (see K_d determinations in Table I and also Figure 4). The slower dissociation of these complexes was not indicative of a greater stability of RT binding to RNA- *vs* DNA-primed templates. When a short RNA or DNA oligonucleotide was hybridized to a long DNA template, binding of the RT to both types of hybrids was about equally stable. Our results also indicated that Mg^{2+}, although not required for the binding of RT to duplex DNA, greatly stabilizes the RT–duplex complexes.

The length of the template strand also influenced the stability of the RT complexes with duplex substrates. DNA oligonucleotide-primed substrates were in general more stable when the template strand was longer. This was true when the template strand was DNA [compare 30D25D with 75D25D (Table I) and also with 66D30D and 72D30D] or RNA (compare 30R25D with 83R30D). This stabilization may result from additional contacts between the enzyme and template strand, when the length of that strand is sufficiently great. The shortest template strands used in these studies were 30 nucleotides in length. In the case of substrates 30D25D and 30R25D, the primer strand was 25 nucleotides in length. Therefore, on the basis of the crystal structure of HIV-RT (Kohlstaedt *et al.*, 1992), the duplex region of these substrates should have been large enough to span from the polymerase active site of the RT to the RNase H active site (a distance of approximately 20 base pairs). The relative instability of binding to the small templates suggests that interactions between the RT and substrate may occur outside of the region that includes the active sites and the space between them.

We questioned whether the presence of an RNA–DNA hybrid region was the primary reason for the enhanced stability of the DNA oligonucleotide-primed RNA substrates. If so, one would expect that an RNA- *vs* DNA-primed DNA template would display more stable binding to the RT. Other evidence, however, suggests that the position of binding on RNA *vs* DNA primers may be quite different, making it hard to predict binding stability. The position of binding to DNA primers tends to be coordinated by the binding of the polymerase activity site of the RT to the 3′-terminal nucleotide of the DNA primer. This conclusion is based on results that show that RT RNase H-directed cleavages on RNA–DNA hybrids occur preferentially at a fixed distance from the 3′ terminus of the DNA primer strand. The distance between the 3′ terminus of the primer and RNase H-directed cleavage remains fixed even as the 3′ terminus of the primer strand is advanced (Furfine & Reardon, 1991). This suggests that the position of the cleavage is determined by the binding of the RT polymerase active site to the primer 3′ terminus. However, work in our laboratory (manuscript in preparation) suggests that binding to some RNAs, annealed as primers, is not coordinated by the polymerase active site binding to the 3′-terminal nucleotide of the RNA. A recent study (Peliska & Benkovic, 1992) also indicates that HIV-RT shows a strong preference to initiate DNA synthesis from a DNA as opposed to an RNA primer. Thus, the RT may recognize the RNA primer as a substrate for degradation, while the DNA primer is recognized as a DNA synthesis initiation site. Our current results demonstrate little difference in the stability of binding to RNA *vs* DNA primers on DNA templates. Evidently the mere presence of the RNA–DNA hybrid does not enhance binding stability. Furthermore, differences in the position or mechanism of binding to the different substrates also appear to have little effect on binding stability.

A comparison of the k_{off} values for 30R25D and 30R30D (Table I) indicates that the 5′ template extension in 30R25D did not contribute to the stability of the RT–substrate complex. It appears that the RT can still bind tightly to the 3′ terminus of the DNA even in a blunt-end conformation. The result was somewhat surprising because the recessed 3′ terminus would be expected to generate a template for DNA synthesis, and a binding site for a DNA polymerase domain. Efficient strand transfer synthesis, as observed *in vitro* (Buiser *et al.*, 1991; Huber *et al.*, 1989), requires that the RT remain bound to the original primer-template after completion of synthesis until transfer occurs. It then continues to remain bound to the 3′ terminus for subsequent synthesis on the new template. This is consistent with stable binding to blunt-ended termini. A recent study addressing the mechanism of strand transfer synthesis (Peliska & Benkovic, 1992) indicates that HIV-RT can add additional non-template-directed nucleotides onto the DNA strand of a blunt-end heteroduplex. This supports the hypothesis that the RT may recognize blunt-end structures as potential incorporation sites.

The initial products generated by HIV-RT RNase H-directed cleavage of the RNA strand of templates 30R25D and 30R30D were different (Figure 2C). Analysis of these products (data not shown) indicated that the initial cleavage site was advanced approximately five nucleotides (the difference in length between 25D and 30D) toward the 5′ end of the RNA strand on template 30R30D. In both cases, the initial cleavages occurred approximately 20 nucleotides behind

(toward the 3′ end of the RNA strand) the 3′-terminal nucleotide of the DNA primer strand (data not shown). These results were consistent with the cleavages having been coordinated by the binding of the RT polymerase domain to the 3′ terminus of the DNA primer on both templates (Furfine & Reardon, 1991; Kohlstaedt *et al.*, 1992). The data additionally suggest that the RT polymerase active site participates in binding contact with blunt-end structures.

It is difficult to compare the kinetic and equilibrium constants that we obtained with those reported by other groups (Huber *et al.*, 1989; Krug & Berger, 1991; Reardon, 1992; Yu & Goodman, 1992). As we have indicated, these values may vary greatly depending on the reaction conditions and templates on which they were determined. Reaction parameters that would be expected to have significant effects include the concentration of salt and the presence or absence of Mg^{2+} during enzyme equilibration with the template. Also the size of the templates and whether they were homo- or heteroduplexes would be significant. Others (Reardon, 1992) have also pointed out that the stability of binding of HIV-RT to duplex nucleic acids shows some sequence dependence. Overall, our values for k_{off}, k_{on}, and K_d are in reasonable agreement with those determined by others. For example, Krug and Berg (1991), by steady-state analysis, using globin mRNA–oligo(dT)$_{15}$ as a substrate for RNase H degradation, calculated k_{on}, k_{off}, and K_d of approximately 2×10^5 M^{-1} s^{-1}, 2×10^{-3} s^{-1}, and 11 nM, respectively. These compare well with our values for 30D25D (in the presence of Mg^{2+}) of approximately 2.9×10^5 M^{-1} s^{-1}, 2.7×10^{-3} s^{-1}, and 9 nM, respectively.

We did not evaluate the effect of Mg^{2+} on the stability of HIV-RT–RNA–DNA complexes. This was because inclusion of Mg^{2+} during the preequilibration phase of our experiments would have allowed the RNase H activity of the RT to degrade the RNA portion of the heteroduplex. Therefore, the stability of the intact complex could not be measured. Since Mg^{2+} stabilizes RT–DNA–DNA complexes, it seems likely that RT–RNA–DNA complexes would also be stabilized, perhaps to a similar extent.

Overall, our results indicate that HIV-RT complexes with DNA oligonucleotide-primed RNA templates are the most stable of the substrates tested. Examination of the K_d values, and a direct competition assay using one pair of homologous substrates (30R25D and 30D25D), showed that binding to DNA-primed RNA templates is of higher affinity than to DNA-primed DNA templates. The slower dissociation rate appeared to be the main contributor to the higher affinity. DNA-primed RNA is the substrate of first-strand DNA synthesis, while DNA-primed DNA is the substrate for second-strand synthesis. The results are consistent with RT binding preferentially and more stably to the nucleic acid structures present during first-strand synthesis as opposed second-strand synthesis. This preference may contribute to a temporal emphasis of first-strand synthesis over second-strand synthesis during replication. Such an ordering seems logical since the product of first-strand synthesis (minus-strand DNA) is the template for second-strand synthesis. Given, however, that each retrovirus virion contains several RT molecules, it is not surprising that portions of both phases of replication occur simultaneously (Varmus & Swanstrom, 1984).

In conclusion, we have shown that binding of HIV-RT to duplex nucleic acids is influenced by at least three factors: (1) The RT binds with higher affinity and greater stability to DNA-primed RNA rather than DNA templates. (2) Template strands, with lengths considerably greater than the distance between the polymerase and RNase H active sites of the RT, bind RT more stably than smaller templates. (3) Mg^{2+} and low salt concentrations stabilize the interaction between RT and nucleic acid duplexes.

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