provided by the covalent linkage of modules. The design criteria that allowed the construction of ZFHD1 included the short length of polypeptide linker that was required to fuse the DNA binding domains and the absence of steric interference between these domains.

Designed transcription factors will be useful for the targeted regulation of specific cellular genes. The use of particular DNA binding domains in a hybrid (or the addition of other domains) may allow a protein to interact with other cellular factors or to be modulated by a particular regulatory pathway. The structure-based design of hybrid transcription factors should facilitate the development of efficient and specific reagents for biological research and gene therapy.

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18. Each model of a hybrid protein (Figs. 1 and 5) was constructed by juxtaposition of portions of two different crystallographically determined protein-DNA complexes. Models were initially prepared by superimposition of the double helices in various registers and were analyzed to see how the polypeptide chains might be connected. Superimposition of sets of phosphates typically gave root mean squared distances of 0.5 to 1.5 Å between corresponding atoms. These distances give some perspective on the error limits involved in modeling and were one of the reasons we chose a flexible linker containing several glycine residues for these initial studies.
19. A fragment encoding Zif268 residues 333 to 390 (*13*), two glycine residues, and Oct-1 residues 378 to 439 (*14*) was generated by polymerase chain reaction (PCR), confirmed by dideoxysequencing, and cloned into the Bam HI site of pGEX2T (Pharmacia) to generate an in-frame fusion to GST. The GST-ZFHD1 protein was expressed by standard methods (*15*), purified on glutathione Sepharose 4B (Pharmacia) according to the manufacturer's protocol, and stored at −80°C in 50 mM tris (pH 8.0), 100 mM KCl, and 10% glycerol. Protein concentration was determined by densitometric scanning of Coomassie-stained SDS–polyacrylamide gel electrophoresis (PAGE)–resolved proteins with the use of bovine serum albumin (BSA) (Boehringer Mannheim) as standard.
20. The probe used for random binding site selection contained the sequence 5′-GGCTGAGTCTGAACGGATCCN$_{25}$CCTCGAGACTGAGCGTCG-3′. Four rounds of selection were done as described (*10*), except that 100 ng of poly[d(I-C)]–poly[d(I-C)] and 0.025% NP-40 were included in the binding reactions. Selections used 5 ng of randomized DNA in the first round and approximately 1 ng in subsequent rounds. Binding reactions contained 6.4 ng of GST-ZFHD1 in round 1, 1.6 ng in round 2, 0.4 ng in round 3, and 0.1 ng in round 4.
21. DNA binding reactions contained 10 mM Hepes (pH 7.9), 0.5 mM EDTA, 50 mM KCl, 0.75 mM dithiothreitol, 4% Ficoll-400, and BSA (300 μg/ml), with the appropriate protein and binding site in a total volume of 10 μl. The concentration of binding site was always lower than the apparent dissociation constant by at least a factor of 10. The PA–Oct-1 POU fusion has been described (*16*). The purified three-finger Zif268 peptide (*1*) was a gift from M. Elrod-Erickson. Reactions were incubated at 30°C for 30 min and resolved in 4% nondenaturing polyacrylamide gels (*16*). Apparent dissociation constants were determined as described (*10*). Probes were derived by cloning of the following fragments into the Kpn I and Xho I sites of pBSKII+ (Stratagene) and excision of the fragments with Asp^{718} and Hind III: 5′-CCTCGAGGTCATTATGGGCGCTAGGTACC-3′, 5′-CCTCGAGGCGCCCATCATTACTAGGTACC-3′, 5′-CCTCGAGGCGCCCACGCCTAGGTACC-3′, and 5′-CCTCGAGGTCATTTGCATACTAGGTACC-3′.
22. The ZFHD1-VP16 expression vector was constructed by cloning of a fragment encoding the epitope Met-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala, ZFHD1, and VP16 residues 399 to 479 (*17*) into the Not I and Apa I sites of Rc/CMV (Invitrogen). Reporter vectors were constructed by cloning of the following fragments into the Xho I and Kpn I sites of pGL2-Promoter (Promega): 5′-GGTACCAGTATGCAAATGACTGCAGTATGCAAATGACCTCGAG-3′, 5′-GGTACCAGGCGTGGGCGCTGCAGGCGTGGGCGCCTCGAG-3′, and 5′-GGTACCAGTAATGATGGGCGCTGCAGTAATGATGGGCGCCTCGAG-3′. The 293 cells were transfected with the use of calcium phosphate precipitation with a glycerol shock as described (*15*). Quantitation of human growth hormone (HGH) production was done with the Tandem-R HGH Immunoradiometric Assay (Hybritech, San Diego, CA) according to the manufacturer's instructions. Cell extracts were made 48 hours after transfection (*15*) and luciferase activity was determined with the use of 10 μl of 100 μl of total extract per 10-cm plate and 100 μl of Luciferase Assay Reagent (Promega) in a ML2250 Luminometer (Dynatech Laboratories, Chantilly, VA), with the use of the enhanced flash program and integration for 20 s with no delay.
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Footprint Analysis of Replicating Murine Leukemia Virus Reverse Transcriptase

Birgitta M. Wöhrl, Millie M. Georgiadis, Alice Telesnitsky, Wayne A. Hendrickson, Stuart F. J. Le Grice*

Replication complexes that contained either murine leukemia virus reverse transcriptase (MLV RT) or a variant reverse transcriptase without a ribonuclease (RNase) H domain (ΔRH MLV RT) were visualized by enzymatic footprinting. Wild-type MLV RT protected template nucleotides +6 to −27, and primer nucleotides −1 to −26 of primers that had first been extended by one or four nucleotides. Although it catalyzed DNA synthesis, ΔRH MLV RT stably bound template-primer only under conditions of reduced ionic strength and protected the duplex portion only as far as position −15. Despite altered hydrolysis profiles, both enzymes covered primarily the template-primer duplex, contradicting recent predictions based on the structure of rat DNA polymerase β.

Even though they catalyze common reactions, retroviral reverse transcriptases (RTs) are structurally diverse. Whereas human, equine, feline, and simian enzymes share a heterodimeric organization of subunits encoded by the RT gene (*1–5*), a subunit of the avian sarcoma-leukosis virus (ASLV) enzyme retains the integrase domain of the gag-pol polyprotein (*6*). The isolated enzyme of the murine leukemia virus (MLV) is a 75-kD monomer, whereas a 150-kD homodimer is proposed to catalyze DNA synthesis (*7*). A structural motif common to RNase H of *Escherichia coli* and MLV RT (α helix III) is also absent from the human immunodeficiency virus (HIV) enzyme (*8*). Such observations illustrate the importance of comparative studies in understanding the evolution of these multifunctional enzymes.

We used chemical (9) and enzymatic probes (*10*) to develop a model of replicating HIV–type 1 (HIV-1) RT that is compatible with the crystallographic data (*11–13*). Hydroxyl radical footprinting (9) and a cocrystal of RT and template-primer (*12*) indicate that ~18 base pairs (bp) of nucleic acid span the DNA polymerase and RNase H catalytic centers. In addition, the distortion of duplex DNA in the cocrystal correlates with a "window" of template and primer nucleotides that are accessible to chemical cleavage. Biochemical and structural models also predict that 4 to 5 bp of substrate occupy the RNase H catalytic center. Results of enzymatic footprinting (*10*) suggest a larger "umbrella" of replicating HIV-1 RT shields template nucleotides +7 to –22 and primer nucleotides –1 to –24, which agrees with structural predictions (*13*) and studies of drug sensitivity as a function of template length (*14*). These examples demonstrate the ability of footprinting techniques to evaluate retroviral replication complexes in the absence of a three-dimensional structure. Here, enzymatic footprinting of wild-type MLV RT and a variant without the RNase H domain (ΔRH MLV RT) (*7*) provided structural information about a second retroviral DNA polymerase in addition to the consequence of eliminating the COOH-terminal domain.

The efficiency of DNA-dependent DNA synthesis catalyzed by wild-type and ΔRH MLV RT (Fig. 1A) (*15*) was first determined on a model template-primer (Fig. 1B). Both efficiently extended the primer to the position at which a chain-terminating dideoxynucleotide triphosphate (ddNTP) was incorporated (Fig. 1C) (*16*). This was important for ΔRH MLV RT, because a hairpin structure near the template 5′ terminus impaired translocation of human and equine enzymes lacking their RNase H domain (*17*). The data of Fig. 1C contrast with a report that demonstrates reduced processivity for ΔRH MLV RT (*7*). To reconcile these discrepancies, we evaluated the efficiency of primer extension in the presence of polyriboadenylate [poly(rA)]-oligo$(dT)_{12-18}$. As the amount of competitor was increased, stalled intermediates became evident in reactions catalyzed by ΔRH MLV RT (Fig. 1D). This indicates the dissociation and capture of ΔRH MLV RT by poly(rA)-oligo$(dT)_{12-18}$.

B. M. Wöhrl and S. F. J. Le Grice, Division of Infectious Diseases, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA.
M. M. Georgiadis, Chemistry Department, Rutgers University, and Waksman Institute, Piscataway, NJ 08855, USA.
A. Telesnitsky, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109–0620, USA.
W. A. Hendrickson, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA.
*To whom correspondence should be addressed.

Replication complexes with wild-type MLV RT were analyzed by deoxyribonuclease (DNase) I footprinting (*18*) after primer extension by one or four nucleotides (+1 and +4 complexes). In recent work (*10*), locating HIV-1 RT on template-primer was aided by intramolecular base pairing near its 5′ terminus which rendered a portion DNase I–sensitive (Fig. 1B) (*10*, *17*). In both complexes, protection of template nucleotides to position –27 was evident. In agreement with earlier work (9, *10*), the hydrolysis profiles differed only in being displaced on the template by the register of DNA synthesis. The template extremity of replicating MLV RT in Fig. 2A is position +6; although this cannot be determined from the DNase I profile, more accurate localization was provided by S1 footprinting. Equivalent complexes that contained HIV-1 RT (*10*) indicated protection of template nucleotides +7 to –22, which may be due to the molecular masses of the two enzymes.

The same experimental conditions did not generate stable replication complexes with ΔRH MLV RT (Fig. 2A). The similarity between DNase I hydrolysis profiles of the naked template-primer duplex (which was freed of RT before hydrolysis) and those that contained ΔRH MLV RT verifies that DNA synthesis was achieved, but was accompanied by dissociation of the truncated polypeptide, in agreement with the data of Fig. 1D. A similar response was noted with RNase H–deficient HIV-1 and E1AV enzymes; that is, both catalyzed DNA synthesis but gave no DNase I footprint (*19*). To enhance the stability of complexes containing ΔRH MLV RT, we removed NaCl from the assay buffer; this did not influence DNA-dependent DNA synthesis (*20*) but permitted stable binding (Fig. 2B). As predicted, enzyme without the RNase H domain covered considerably less of the template-primer duplex [the footprint of wild-type MLV RT was unaltered at reduced ionic strength (*21*)]. The upstream extremity of a +1 replication complex was relocated to position –15, which is 12 nucleotides shorter than a complex containing the wild-type enzyme (Fig. 2A). Furthermore, the DNase I footprint was interrupted at positions –9 and –10, which remained accessible for hydrolysis. The +1 and +4 replication complexes that contained ΔRH MLV RT were qualitatively identical, including having enhanced accessibility around positions –9 and –10. In spite of an altered interaction of ΔRH MLV RT with the template-primer duplex, the hairpin structure on the template remains DNase I susceptible, which suggests that interactions involving the NH_2-terminus were not altered by eliminating the RNase H moiety.

Interaction of each RT with the DNA primer is shown in Fig. 3. The extremity of a +1 replication complex that contains wild-type MLV RT was estimated to be at position –26. This location corresponds well with the position of the enzyme on the DNA template (Fig. 2A). In contrast, the hydrolysis profile of an equivalent complex containing ΔRH MLV RT extended to position –15. Primer nucleotides –9 and –10 showed no DNase I reactivity, even though template nucleotides –9 and –10 were accessible (Fig. 2B). Also, position –23 on the primer had enhanced DNase I sensitivity but the reason for this is unclear. Other

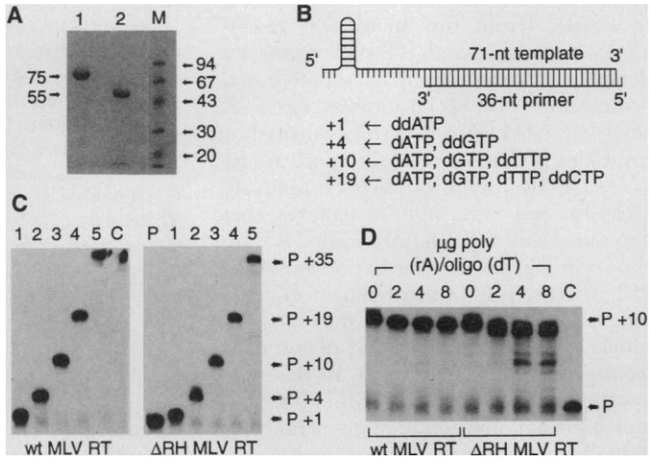
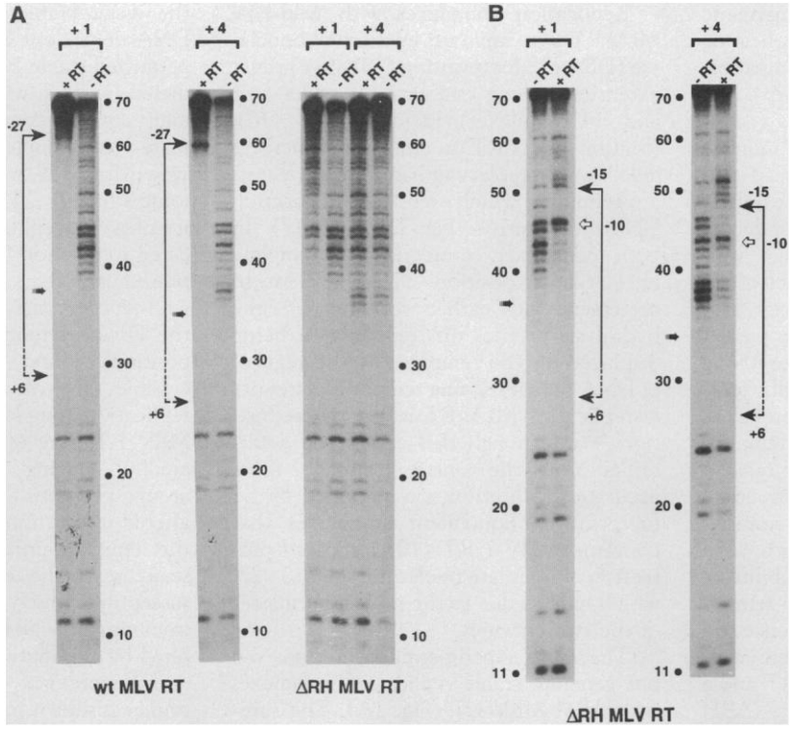


Fig. 1. (**A**) Analysis of purified wild-type (lane 1) and ΔRH MLV RT (lane 2) by SDS–polyacrylamide gel electrophoresis. Lane M, protein molecular size standards in kilodaltons. (**B**) Schematic representation of the 71-nt–36-nt template-primer used for studying replication complexes. The hairpin structure near the template 5′ terminus was determined by partial DNase I and S1 hydrolysis (*10*). The dNTP-ddNTP combinations that produced "arrested" replication complexes are indicated. (**C**) DNA polymerase activity of wild-type and ΔRH MLV RT. The designations P +1, P +*n* represent primer extension reactions outlined in (B). Lane P, unextended primer; lane C, end-labeled template. The P +35 product is obtained in the absence of chain termination. (**D**) Substrate challenge experiments with wild-type and ΔRH MLV RT. With the template-primer combination of (B), the capacity of each enzyme to complete a +10 primer extension reaction in the presence of increasing amounts of poly(rA)-oligo$(dT)_{12-18}$ was determined. Lane C, unextended primer.



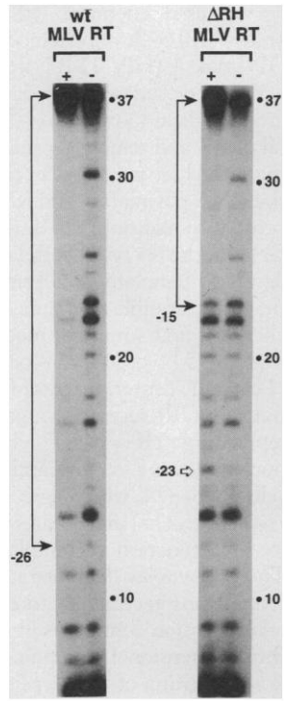


Fig. 2 (left). DNase I footprinting of +1 and +4 replication complexes containing wild-type and ΔRH MLV RT. Template DNA was labeled at its 5′ terminus with [^{32}P]. In (**A**), complexes were prepared in a buffer containing 80 mM NaCl. The boundaries of +1 and +4 replication complexes containing wild-type (wt) MLV RT are indicated, within which the arrow indicates incorporation of the chain-terminating ddNTP (defined here as position −1). (**B**) Replication complexes containing ΔRH MLV RT were analyzed in buffer lacking NaCl. Regions of the replication complex with altered DNase I sensitivity are indicated by the white arrow. The extremity of both enzymes on the single-stranded template is indicated by a broken line, because this is derived from the S1 footprinting data of a later experiment. **Fig. 3 (far right).** DNase I hydrolysis profiles of primer DNA of +1 replication complexes containing MLV RT variants. Primer DNA was labeled at the 5′ terminus. Hyperaccessibility to DNase I at position −23 in the presence of ΔRH MLV RT is indicated by the white arrow.

than these differences, DNase I footprinting of ΔRH MLV RT demonstrates that protection of ~12 bp of the template-primer duplex (−16 to −27) is lost as a consequence of removing the RNase H domain.

The cocrystal of HIV-1 RT and template-primer (*9*–*13*) predicts that the fingers subdomain of the MLV enzymes could interact with six to seven template nucleotides beyond the DNA polymerase catalytic center. [From the anatomical resemblence to a right hand, RT subdomains are denoted "fingers," "palm," "thumb," and "connection" (*11*).] The interaction with single-stranded DNA is better evaluated by resistance of the nucleotides to nuclease S1 (*22*). S1 hydrolysis profiles of template DNA in +4 replication complexes that contained wild-type and ΔRH MLV RT are shown in Fig. 4A. Similar to previous work (*10*), this revealed primarily single-stranded template nucleotides between the template-primer duplex and the region of intramolecular base pairing (Fig. 1B). In the presence of wild-type and ΔRH MLV RT, several template nucleotides were wholly or partially S1 resistant. With this strategy, the extremity of both enzymes was located six nucleotides ahead of the position of chain termination. The location of the two MLV enzymes at position +6 agrees with data from complexes containing HIV-1 RT (*10*, *14*) which indicate that the β3-β4 hairpin of its p66 fingers subdomain interacts with template nucleotides +3 to +6. More importantly, data of Fig. 4 show that both MLV enzymes accommodate equivalent amounts of template during DNA synthesis. The combined MLV RT footprinting data are summarized in Fig. 5 and compared with complexes that contain the HIV-1 enzyme.

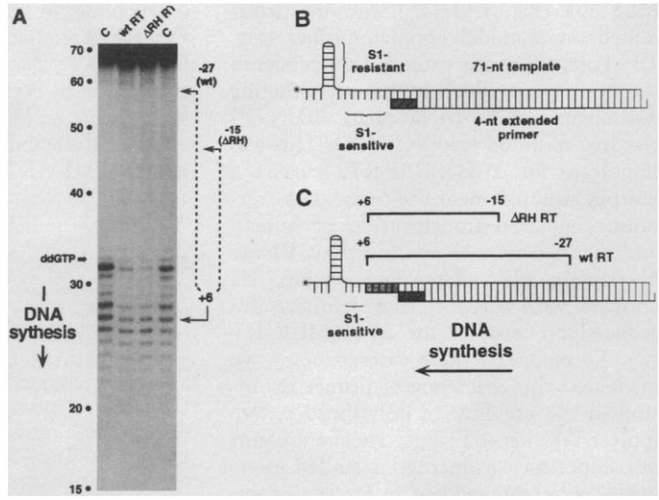


Fig. 4. (**A**) Susceptibility of +4 replication complexes containing wild-type or ΔRH MLV RT to nuclease S1 digestion. Template DNA was labeled at its 5′ terminus. Lanes C, control S1 hydrolysis of template on which the primer was extended by four bases. (**B**) Schematic S1 hydrolysis profile of template DNA on which the primer was extended by four bases (striped, shaded box). The S1-resistant hairpin structure was deduced from previous work (*10*). (**C**) Schematic S1 hydrolysis profile of +4 replication complexes containing MLV RT variants. The shaded box on the template represents nucleotides protected from S1 hydrolysis in the presence of RT.

Structural studies (*9*–*13*) indicate that the fingers subdomain of p66 HIV-1 RT contacts primarily its palm, whereas the RNase H domain contacts the extended thumb of p51. Our notion that the structural integrity of the MLV RT fingers subdomain (and interaction with the template) is not compromised by elimination of the RNase H domain is therefore not unreasonable. Replication complexes containing HIV-1 RT suggested that its p66 fingers

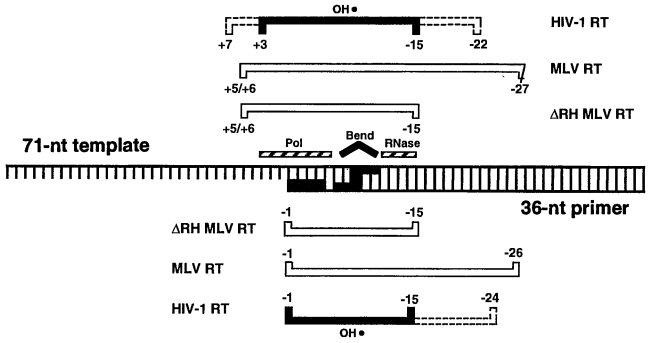


Fig. 5. Comparison of replication complexes containing MLV and HIV-1 RT. A +4 complex catalyzed by the three enzymes is presented (primer extension is indicated by a shaded box). For HIV-1 RT, filled boxes indicate footprints derived from hydroxyl radical footprinting (*9*), whereas broken lines extending this are the limits of the complex determined by enzymatic footprinting (*10*). Delineation of the DNA polymerase (Pol) and RNase H active centers (RNase), in addition to a 45° bend in substrate, is according to (*9*) and (*12*). Filled boxes within the duplex represent template and primer nucleotides of the complex accessible to chemical cleavage (*9*).

protect template nucleotides as far as +7 (*10*). Whereas S1 footprinting provides only low-resolution information, the crystal structure of a proteolytic fragment of MLV RT indicates that a tighter angle exists between its fingers and palm (*23*), which suggests that MLV RT extends over a slightly smaller portion of the template. Although interactions with the single-stranded template are unaffected, the template-primer duplex between –16 and –27 is DNase I sensitive in complexes that contain ΔRH MLV RT.

These observations are consistent with biochemical and structural data available for HIV-1 RT. Chemical footprinting (*9*) predicts that within 18 template and 15 primer nucleotides embraced by the replicating enzyme, duplex nucleic acid between –12 to –15 occupies the RNase H catalytic center. Subsequent structural analysis (*13*) and DNase I footprinting (*10*) predict that an additional 5 to 6 bp may be shielded by the RNase H domain. These combined observations suggest that the HIV-1 RNase H domain could cover the primer-template duplex from –12 to –22, which agrees with the loss of protection over 12 bp of the template-primer duplex found here. The elimination of the MLV RNase H domain also alters DNase I sensitivity at template positions –9 and –10, which remain accessible within a larger region of protection. Current models of replicating HIV-1 RT (*9*–*13*) suggest positions –9 and –10 of the template-primer duplex lie in the nucleic acid–binding cleft between the catalytic centers. Elimination of the RNase H domain may compromise the architecture of this cleft or constraints imposed on the template-primer duplex. Duplex DNA was shown to adopt A- and B-form geometry in the DNA polymerase and RNase H catalytic centers, respectively (*12*). The absence of an RNase H domain may relieve constraints on A-form DNA, leaving the DNA polymerase catalytic; also, a 45° bend in nucleic acid between the catalytic centers (*12*) may not be facilitated. Such rearrangements may render the template DNase I sensitive at positions –9 and –10 and alter the affinity of ΔRH MLV RT for template-primer.

These data confirm and extend structural features of HIV-1 RT (*9*–*14*), but contradict other reports (*24*, *25*). The structure of a ternary complex containing rat DNA polymerase β predicts that during DNA-dependent DNA synthesis, HIV RT occupies primarily the template and not the template-primer duplex. In contrast, we find that MLV RT shields the template-primer duplex, to an extent that is influenced by the elimination of the RNase H domain. Because we observe equivalent occupancy of the template-primer duplex with HIV and E1AV RT (*10*, *26*), the source of the enzyme does not account for these discrepancies. In summary, our results support the disposition of RT on template-primer previously reported (*11*, *12*).

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16. DNA-dependent DNA polymerase activity was determined on a 71–nucleotide (nt)–36 nt template-primer combination (Fig. 1B) (*10*, *17*) in a buffer of 60 mM tris-HCl, 75 mM NaCl, 7.5 mM $MgCl_2$, 5 mM DTT, and 0.05% NP-40. RT (100 ng) was incubated with 0.5 to 1.0 pmol of template-primer for 2 min at 37°C, after which a dNTP-ddNTP cocktail was added (final concentrations of 50 and 500 μM, respectively) that was designed to permit primer extension by 1, 4, 10, and 19 nucleotides. After 20 min at 37°C, DNA synthesis was terminated, and the products were analyzed by high-resolution gel electrophoresis and autoradiography (Du Pont "Reflection"). For substrate challenge experiments, 25 ng of enzyme was incubated with template-primer in polymerization buffer. After 5 min at 37°C, a dNTP-ddNTP mix that allows primer extension by 10 nucleotides was added, together with increasing amounts of poly(rA)-oligo$(dT)_{12–18}$. DNA polymerase products were analyzed as described above.
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