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Truncating α-Helix E′ of p66 Human Immunodeficiency Virus Reverse Transcriptase Modulates RNase H Function and Impairs DNA Strand Transfer*

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Madhumita Ghosh‡§, Kathryn J. Howard‡, Craig E. Cameron¶‖, Stephen J. Benkovic¶, Stephen H. Hughes, and Stuart F. J. Le Grice‡ ‡‡**

*From the ‡Division of Infectious Diseases, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, the ¶Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802-6300, and **ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201*

The properties of recombinant p66/p51 human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) containing C-terminal truncations in its p66 polypeptide were evaluated. Deletion end points partly or completely removed α-helix E′ of the RNase H domain (p66Δ8/p51 and p66Δ16/p51, respectively), while mutant p66Δ23/p51 lacked αE′ and the β5′-αE′ connecting loop. Although dimerization and DNA polymerase properties of all mutants were not significantly different from those of the parental enzyme, p66Δ16/p51 and p66Δ23/p51 RT lacked ribonuclease H (RNase H) activity. In contrast, RT mutant p66Δ8/p51 retained endonuclease activity but lacked the directional processing feature of the parental enzyme. Despite retaining full endoribonuclease function, p66Δ8/p51 RT barely supported transfer of nascent (−)-strand DNA between RNA templates representing the 5′ and 3′ ends of retroviral genome, shedding light on the requirement for the endonuclease and directional processing functions of the RNase H domain during replication.

In contrast to the DNA polymerase activity of human immunodeficiency virus reverse transcriptase (HIV RT),[1] antiviral drugs targeted to its ribonuclease H (RNase H) domain are scarce (1, 2), despite documentation that this activity is essential for viral infectivity (3, 4). Lack of (i) detailed structural information, (ii) defined substrates, and (iii) pure recombinant enzyme for *in vitro* analysis have undoubtedly hampered past studies. However, these issues have been largely resolved, thereby offering RNase H activity as another avenue of therapeutic intervention in our efforts to curtail the spread of HIV infection and devastating consequences of acquired immunodeficiency syndrome (AIDS). For example, the three-dimensional structure of HIV-1 RNase H is now available as an isolated domain (5) and a component of the parental p66/p51 heterodimer (6, 7). Furthermore, the availability of synthetic RNA now allows construction of model substrates mimicking steps in the replication cycle invoking RNase H activity, *e.g.* DNA strand transfer (8, 9), generating the polypurine tract primer for (+)-strand synthesis (10) and removing the (−)- and (+)-strand primers from nascent DNA (11, 12). These advances have benefited from efficient methods of expression and preparation of recombinant HIV-1 and HIV-2 RT free of contamination by bacterial enzymes (13–15).

The observation that an initial endonucleolytic cut is accompanied by processing of the RNA template (although the latter was originally defined as exonuclease activity (16), we believe that the term "directional processing" more accurately reflects these events), and the ability of human and murine enzymes to hydrolyze double-stranded RNA (RNaseH* activity (17–19), indicates an unexpected versatility for the 120-residue C-terminal domain. Understanding this expanded repertoire of nuclease functions should prove beneficial to drug development programs. Several lines of evidence implicate structural elements at the extreme C terminus of the RNase H domain in the architecture and activity of the p66/p51 heterodimer. Although disordered in the isolated HIV-1 and HIV-2 RNase H domains (20), the p66 β5-αE′ connecting loop (residues 537–543) has been shown to interact with αH and the αI-αJ connecting loop of the p51 thumb subdomain (21). The β5′-αE′ connecting loop contains the invariant His^{539}, which has been shown for bacterial and retroviral RNases H to play a role in substrate binding and catalysis (22–27). The last element of p66 RT, α-helix E′ (residues 544–555), together with p51 helices αH and αI, may also provide a "floor and wall" of the nucleic acid binding cleft immediately adjacent to the RNase H catalytic center.[2] Finally, α helix E′ contains the conserved Asp^{549}, which has been implicated by Davies *et al.* (5, 20) in divalent metal ion coordination. The importance of these elements prompted us to analyze heterodimer RT containing minor C-terminal truncations in its p66 subunit. Our rationale was also based on analysis of reconstituted heterodimer HIV-1 RT containing a C-terminally deleted p51 subunit (28). Elimination of 13 p51 residues compromised tRNA binding in the reconstituted heterodimer, while other functions were unaffected. Extending the deletion end point a further 6 residues influenced both the processivity of DNA synthesis and tRNA binding, while p51 RT lacking 25 residues failed to dimerize.

Here, we purified and evaluated heterodimer HIV-1 RT whose p66 subunit was truncated by 8 (p66Δ8/p51), 16 (p66Δ16/p51), or 23 residues (p66Δ23/p51). In p66Δ8/p51 RT,

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‡‡ To whom correspondence and reprint requests should be addressed. Tel.: 216-368-2034; Fax: 216-368-2034.

[1] The abbreviations used are: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; nt, nucleotides; RNase H, ribonuclease H; RT, reverse transcriptase.

[2] C. Tantillo and E. Arnold, personal communication.

α-helix E′ lacks three residues, while the entire α-helix (including the invariant Asp^{549}) is absent in p66Δ16/p51. p66Δ23/p51 RT lacks α-helix E′ and the β5′-αE′ connecting loop, the latter of which contains the invariant His^{539}. Purified enzymes showed similar levels of RNA- and DNA-dependent DNA synthesis on defined heteropolymeric template-primers. However, while p66Δ23/p51 and p66Δ16/p51 were devoid of RNase H activity, mutant p66Δ8/p51 retains endoribonuclease function. Loss of directional processing activity coincides with a sharp reduction in the efficiency with which mutant p66Δ8/p51 mediates transfer of nascent DNA between RNA templates, indicating its necessity at this step in retroviral replication (29).

EXPERIMENTAL PROCEDURES

Reconstitution and Purification of p66/p51 RT Mutants—p66 C-terminal deletion derivatives described by Hizi *et al.* (30) were used in this study. Mutant CT-8 terminates at Val^{552}, while CT-16 and CT-23 terminate at Gly^{544} and Pro^{537}, respectively. Using procedures developed in our laboratory (28, 31–34), p66 mutants were reconstituted with a polyhistidine-extended p51 subunit by combining the appropriate bacterial homogenates (31). Reconstituted enzymes were purified by a combination of metal chelate (Ni^{2+}-nitrilotriacetic acid-Sepharose) and ion exchange chromatography (DEAE-Sepharose and S-Sepharose). Purified enzymes were judged free of contaminating nucleases by incubation with radiolabeled, single-stranded RNA and duplex DNA. All enzymes were stored at −20 °C in a 50% glycerol-containing buffer (34).

Determination of DNA Polymerase Activities—RNA- and DNA-dependent DNA polymerase activities were determined by programmed synthesis on heteropolymeric template-primers (Fig. 1). DNA-dependent DNA synthesis used a 71-nt template-36-nt primer combination (35, 36) and dNTP/ddNTP mixtures permitting extension by 1, 4, 10, or 19 nucleotides. Reaction mixtures (20 μl) contained 50 mM Tris/HCl, pH 8.0, 6 mM $MgCl_2$, 0.05% (v/v) Triton X-100, 80 mM NaCl, 5 mM dithiothreitol, 0.1–0.2 pmol of template-primer, 0.5–1.0 pmol of wild type or mutant RT, 50 μM dNTPs, and 500 μM ddNTP. Reactions were initiated by addition of RT and allowed to proceed for 10 min at 37 °C.

RNA-dependent DNA polymerase activity was determined in a similar manner on an analogous 90-nt template-36-nt primer, with the exception that the template was extended at its 5′ terminus by 19 nucleotides. "Programmed" primer extensions were therefore identical in both cases, while full-length products from RNA- and DNA-dependent DNA synthesis were 90 and 71 nt, respectively. Reaction products were fractionated by high voltage gel electrophoresis through 10% or 12% polyacrylamide gels containing 7 M urea in Tris/borate/EDTA buffer (36). After drying, gels were subjected to autoradiography, using the DuPont "Reflections" system.

Qualitative Evaluation of RNase H Activity—The 90-nt template-36-nt primer of the previous section was also used to evaluate RNase H function by relocation of radiolabel to the template 5′ terminus. RNase H activity was determined either in the absence of DNA synthesis or following extension of the primer by 10 nucleotides. Hydrolysis products were fractionated by high voltage electrophoresis and analyzed as described above. Product size was determined by co-electrophoresis of both a partial RNase A and alkaline hydrolysates of the radiolabeled RNA template. Heparin challenge experiments were performed in the presence of 1 mg/ml heparin.

Determination of Strand Transfer Capacity—The efficiency of DNA strand transfer was determined according to Peliska and Benkovic (9). The sequences of oligonucleotides for these experiments were: 5′-AGAGCTCCCAGGCTCAGATC-3′ (20-nt DNA primer), 3′-UCUCGAGGGUCCGAGUCUAGACCAGAUUGGUCUCUCUGGGG-5′ (40-nt donor RNA template), and 3′-ACCAGAUUGGUCUCUCUGGGUCAUGUCCGUUUUUCGUCGAG-5′ (41-nt acceptor RNA template).

Strand transfer reactions were performed in a buffer of 50 mM Tris-HCl, pH 8.0, 75 mM KCl, 1 mM dithiothreitol, 0.1% Triton X-100, 7 mM $MgCl_2$, 100 μM dNTPs, 200 nM 5′-end-labeled 20-nt DNA/40-nt RNA, 480 nM acceptor RNA template, and 200 nM p66/p51 HIV-1 RT. Reactions were initiated at 37 °C by addition of RT; at the times indicated, samples were withdrawn and DNA synthesis was terminated by supplementing with EDTA to a final concentration of 110 mM. Reaction products were fractionated by high voltage electrophoresis through denaturing 15% polyacrylamide gels, visualized with a Molecular Dynamics PhosphorImager, and quantified with ImageQuant software (provided by the supplier).

DNase I Footprinting of Replication Complexes—The DNase I footprinting protocol of Schmitz and Galas (37) was followed, with minor modifications. 200 ng of wild type or mutant RT (1.7 pmol) was incubated with ~1 pmol of end-labeled template-primer in a 15-μl reaction mixture containing 10 mM Tris/HCl, pH 8.0, 6 mM $MgCl_2$, 80 mM NaCl, 5 mM dithiothreitol, and the appropriate dNTP/ddNTP mixture (final concentrations 50 and 500 μM, respectively). After 20 min at 37 °C, 1 unit of DNase I (Boehringer Mannheim) was added, and, 30 s later, the reaction was terminated by adding an equal volume of phenol/chloroform/isoamyl alcohol. Nucleic acid in the aqueous phase was precipitated in the presence of glycogen, and the dried sample was resuspended in 10 μl of a urea-based gel loading buffer. Hydrolysis products were fractionated by high voltage electrophoresis and visualized by autoradiography. Since replication complexes generated increasing amounts of duplex DNA substrate, it was necessary to prepare control DNase I digests for "+4" and "+10" primer extension reactions. To achieve this, the appropriate replication complex was prepared with wild type RT, then freed of enzyme by phenol extraction. Extended DNA substrates were recovered by ethanol precipitation and subjected to partial DNase I hydrolysis as described above.

RESULTS

Qualitative Evaluation of DNA Synthesis—Recent reports indicate that retroviral RTs with quantitatively similar DNA polymerase activities differ significantly in processivity (36, 38). Therefore, we elected initially to determine DNA- and RNA-dependent DNA polymerase activities of our mutants on heteropolymeric template-primer combinations. The substrates (Fig. 1) use the same 36-nt DNA primer, while their templates differ in length by 19 nt at their 5′ termini. "Programmed" DNA synthesis reactions could then be used to evaluate the efficiency of equivalent primer extension reactions directed by RNA and DNA templates. The results of this analysis are presented in Fig. 2.

Comparison of wild type RT (Fig. 2*A*, *panel iv*) and the three deletion mutants (Fig. 2*A*, *panels i–iii*) indicates that removal of as many as 23 residues from the RNase H C terminus was tolerated without any noticeable alteration in the efficiency of DNA-dependent DNA synthesis. Fig. 2*B* illustrates RNA-dependent DNA synthesis on a related template-primer and provides a similar result. Analysis of the RNA template with nuclease S1 (specific for single-stranded nucleic acid), RNase CV (specific for double-stranded RNA), and RNase A (which cleaves after pyrimidine residues in single-stranded RNA) indicate that extensive intramolecular base pairing is adopted.[3] However, this presents no major impediment to the translocating enzymes, evidenced by the lack of stalled intermediates. Curiously, the exception to this is wild type p66/p51 RT, which (*a*) initiates cDNA synthesis less efficiently and (*b*) experiences minor stalling at the P+3 and P+4 positions. These features are not reconciled by increasing the enzyme:substrate ratio (data not shown), suggesting that initiation of RNA-dependent DNA synthesis may be enhanced by minor incursions into the C-terminal RNase H domain. More importantly, the data of Fig. 2*B* contradict previous observations of Hizi *et al.* (30), who determined that the RNA-dependent DNA polymerase activity of their p66 mutant CT-23 was ~4% of that derived from the wild type enzyme, while that of mutants CT-16 and CT-8 was 45% and 60%, respectively. Retention of both RNA- and DNA-dependent DNA polymerase function in heterodimer RT containing these p66 subunits may reflect a more acceptable conformation in the singly-mutated heterodimer as opposed to the doubly-mutated, truncated homodimers of Hizi *et al.* (30).

RNase H Activity Is Modulated by the Extent of p66 Deletion—As indicated under "Experimental Procedures," the template-primer of Fig. 1*B* could be adapted for qualitative evaluation of RNase H function by relocating radiolabel to the 5′ terminus of the RNA template. This substrate was used to determine RNase H activity prior to DNA synthesis (0 complex)

[3] N. M. Cirino and S. Le Grice, unpublished data.

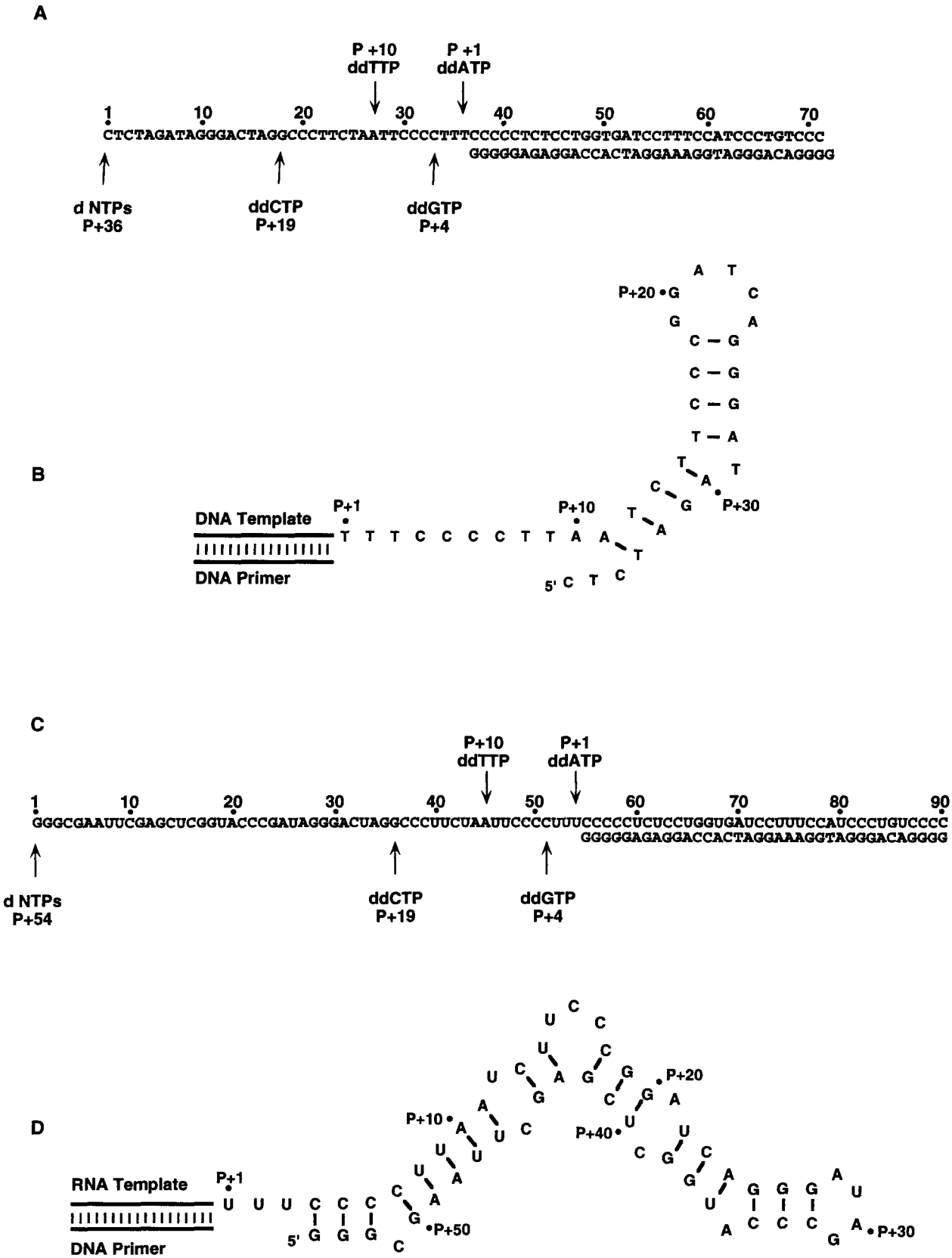

FIG. 1. **Sequence of template-primers used for analysis of DNA- (*A*) and RNA-directed DNA synthesis (*C*).** Both templates make use of the same 36-nt DNA primer and are also homologous over 31 bases immediately adjacent to the template-primer duplex. The positions at which incorporation of the respective ddNTP arrests DNA synthesis is indicated. *B* and *D*, intramolecular base-paired structures assumed by the single-stranded DNA and RNA templates, respectively. *Structure B* was derived by sensitivity to DNase I and S1, while RNase A, RNase CV, and S1 were used to derive *structure D*.

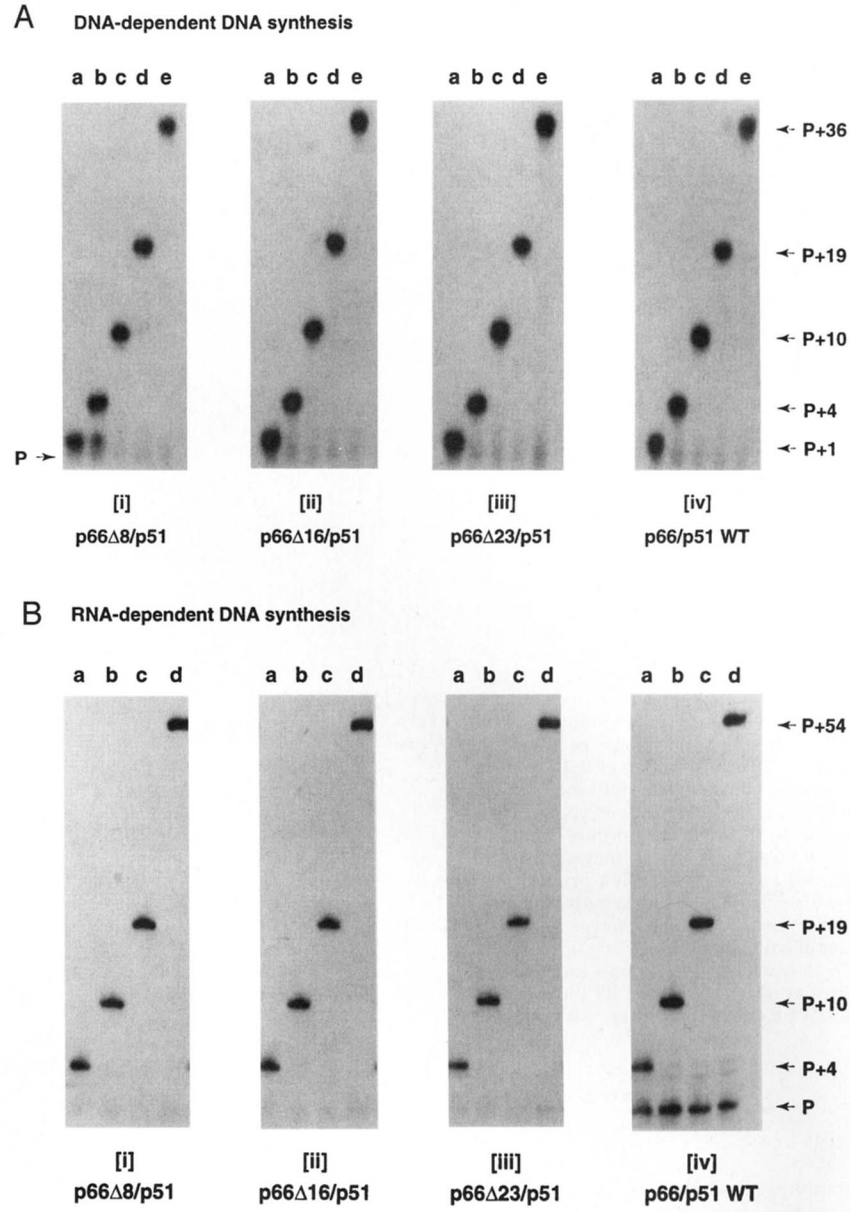

FIG. 2. **Qualitative analysis of DNA polymerase activities of RT mutants.** *A*, DNA-dependent DNA polymerase activity. For each panel, *lanes a–d* represent primer extension by 1, 4, 10, and 19 nucleotides, respectively, while *lanes e* represent DNA synthesis in the absence of chain termination. *P* designates the migration position of the unextended primer. *B*, RNA-dependent DNA polymerase activity. The P+1 extension reaction was omitted from this analysis. *Lanes a–c* represent primer extension by 4, 10, and 19 nucleotides, respectively, while *lanes d* indicate DNA synthesis in the absence of chain termination. Note the accumulation of unextended primer and stalled P+3/P+4 products in reactions catalyzed by wild type RT.

or following addition of 10 nt to the DNA primer (+10 complex), the latter of which allowed us to visualize coordination of the DNA polymerase and RNase H functions.

Fig. 3*A*, *panel i*, illustrates RNase H activity of wild type RT in the absence of polymerization and in response to heparin challenge. In the absence of heparin, where dissociation and rebinding is permitted, cleavage products of 62–64 nt are evident, together with minor amounts of a 71-nt RNA (*lane 1*). Assuming RT locates itself over the 3′ terminus of the DNA primer (9), the 71-nt product implies cleavage at position −17, while the 62- and 64-nt products indicate cleavage at position −8 and −10, respectively. Prebinding RT to template-primer, followed by addition of Mg^{2+} and heparin (which "traps" free and dissociated enzyme, allowing a single binding event to be monitored) reverses this pattern, *i.e.* cleavage at −17 predominates, while those at −8 and −10 are minimized (*panel i*, *lanes 3–5*). Since the 62-nt/64-nt products fail to accumulate over an extended period of heparin challenge (*lanes 3–5*), the data of Fig. 3*A*, *panel i*, indicate that initial cleavage at position −17 is followed by directional processing of the RNA template (by a second enzyme) to around position −8, at which point it most likely dissociates from the DNA primer.

In Fig. 3*A*, *panel ii*, template-primer was incubated with each RT in the absence of dNTPs and heparin. While p66Δ16/p51 and p66Δ23/p51 RT failed to hydrolyze the substrate, mutant p66Δ8/p51 retained significant activity. However, when compared to the data of *panel i*, the products of this reaction (71 and 69 nt) suggested endonuclease activity but an absence of directional processing. This notion was supported by coordinating RNase H activity with RNA-dependent DNA synthesis. Under conditions where the primer was extended by 10 nt (Fig. 2*B*), wild type enzyme gives rise to RNase H cleavage products between 63 and 53 nt (*panel iii*). Relocation of RT to the primer terminus of a +10 replication complex implies RNase H hydrolysis between positions −19 and −9. Although this RNase H hydrolysis profile is complicated by molecules which fail to extend the primer and others which stall at the P+3 and P+4 positions (see Fig. 2*B*), locating the extremity of RNase H cleavage at position −9 is in keeping with RNase H function in the absence of DNA synthesis. In contrast, the RNase H products in a +10 replication complex catalyzed by mutant p66Δ8/p51 (63 and 62 nt) correspond to cleavage at −18/−17, again suggesting predominantly endonuclease activity. Furthermore, the absence of cleavage products arising from stalled enzymes is in keeping with data of Fig. 2*B*,

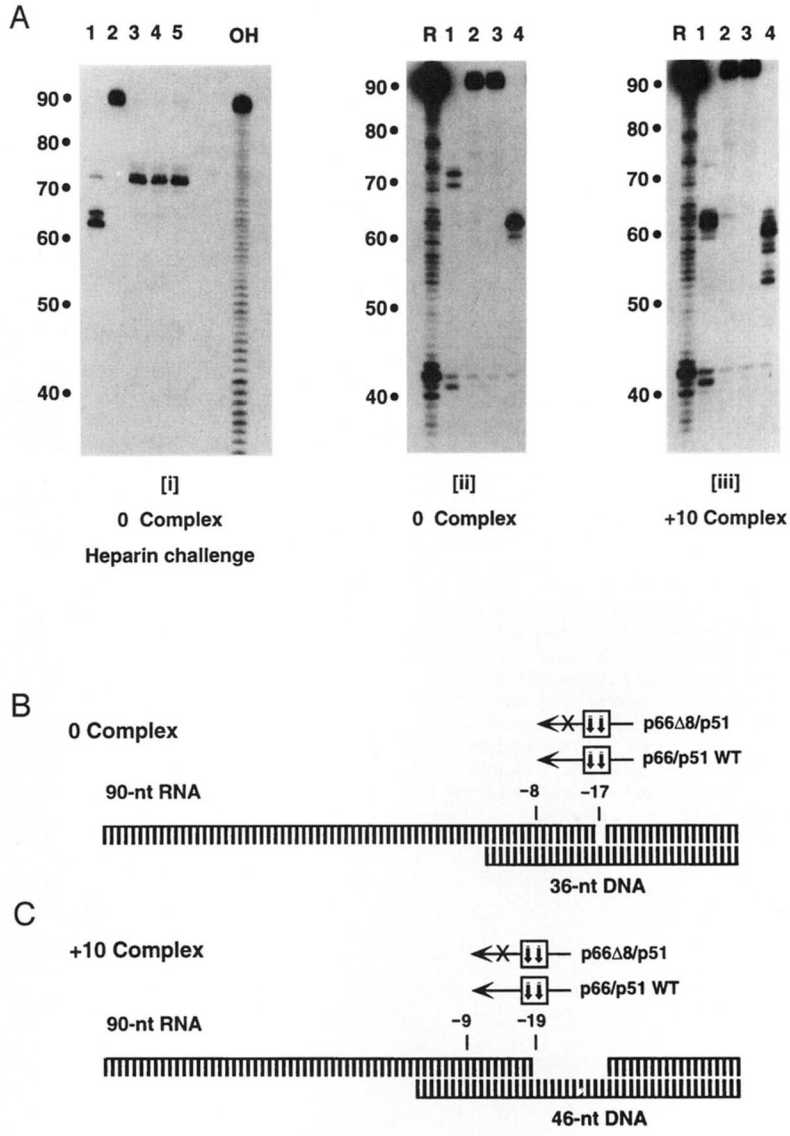

FIG. 3. **RT mutant p66Δ8/p51 retains endoribonuclease activity.** *A*, determination of RNase H activity on the 90-nt RNA-36-nt DNA template-primer of Fig. 1. *Panel i*, effect of heparin on RNase H activity in the absence of DNA synthesis. All assays contained radiolabeled substrate in a buffer lacking Mg^{2+}, which was added later to initiate hydrolysis. *Lane 1*, RNase H activity in the absence of heparin. *Lane 2*, preincubation of RT with heparin. In *lanes 3–5*, RT was incubated with template-primer prior to addition of heparin and Mg^{2+}, and aliquots of the reaction were analyzed after 30 s (*lane 3*), 2 min (*lane 4*), and 10 min (*lane 5*). *OH*, alkaline hydrolysis ladder of the RNA template for determination of product lengths. *Panel ii*, RNase H activity of wild type and mutant RT in the absence of DNA synthesis. *Lane R*, partial RNase A hydrolysis profile of the single-stranded RNA template. *Lane 1*, p66Δ8/p51 RT; *lane 2*, p66Δ16/p51 RT; *lane 3*, p66Δ23/p51 RT; *lane 4*, wild type p66/p51. *Panel iii*, RNase H activity of wild type and mutant RT following primer extension by 10 nucleotides. Lane notations are as in *ii*. *B*, schematic representation of the RNase H activities of wild type RT and mutant p66Δ8/p51 in the absence of DNA synthesis (*upper*) and following primer extension by 10 nucleotides (*lower*, indicated by the *shaded portion* of the DNA primer). For both replication complexes, the endonuclease and directional processing activities of wild type RT are designated by the *arrows*, while *boxed arrows* indicate the sites at which p66Δ8/p51 RT functions as solely an endoribonuclease.

implying that the RT mutant p66Δ8/p51 traverses the RNA template more efficiently than the wild type enzyme.

Endoribonuclease Function Supports Inefficient DNA Strand Transfer—Data in Fig. 3 suggest that wild type RT, located over the primer 3′ terminus, cleaves the RNA template between position −17 and −19, after which it is processed as far as position −8. These activities can be likened to "polymerase-dependent" and "polymerase-independent" RNase H activities proposed by Peliska and Benkovic (9) to mediate transfer of (−)-strand DNA between RNA templates. Upon reaching the RNA 5′ terminus, their model proposes that RT is positioned in a manner permitting the RNase H domain to cleave ~17 nt behind the primer terminus. This event is followed by "polymerization-independent" RNase H activity, which cleaves to position −8, concomitant with which is a rise in strand transfer activity. If directional processing is related to the polymerization-independent mechanism, its absence in mutant p66Δ8/p51 might have consequences for DNA strand transfer.

Fig. 4*A* indicates components of a model strand transfer system. Initially, RT extends a 20-nt DNA primer to the 5′ terminus of the 40-nt donor RNA template. RNase H hydrolysis of the latter facilitates transfer of nascent, 40-nt DNA to a 41-nt acceptor RNA template dictating further synthesis of a 61-nt product. A quantitative evaluation of DNA strand transfer via accumulation of the 61-nt product is presented in Fig. 4*B*. Following an initial lag, wild type RT supports efficient transfer and synthesis of the 61-nt DNA product. In contrast, strand transfer in a reaction catalyzed by mutant p66Δ8/p51 is reduced to 3–4% and only after a considerably longer lag (~10 min). Inspection of the original phosphorimage indicated that the reduction in p66Δ8/p51 RT-catalyzed strand transfer was not a consequence of reduced cDNA synthesis, since the 40-nt intermediate accumulated to the same level with both enzymes (data not shown). The data of Fig. 4*B* rather suggest that endonuclease activity of p66Δ8/p51 RT, upon reaching the 5′ terminus of the donor RNA template, generates a 17-nt RNA which remains stably hybridized to nascent DNA. The requirement for directional processing to complete DNA transfer implicates a specific role for this RNase H function in facilitating transfer of nascent DNA between (or within) strands of the retroviral genome during replication.

Analysis of Replication Complexes Containing RT Mutants—In addition to the functional significance of α-helix E′ of the RNase H domain, this element may contribute to interactions between p66/p51 RT and template/primer.[4] This was addressed by DNase I footprinting of DNA-dependent DNA synthesis complexes containing wild type and mutant enzymes.

[4] E. Arnold, personal communication.

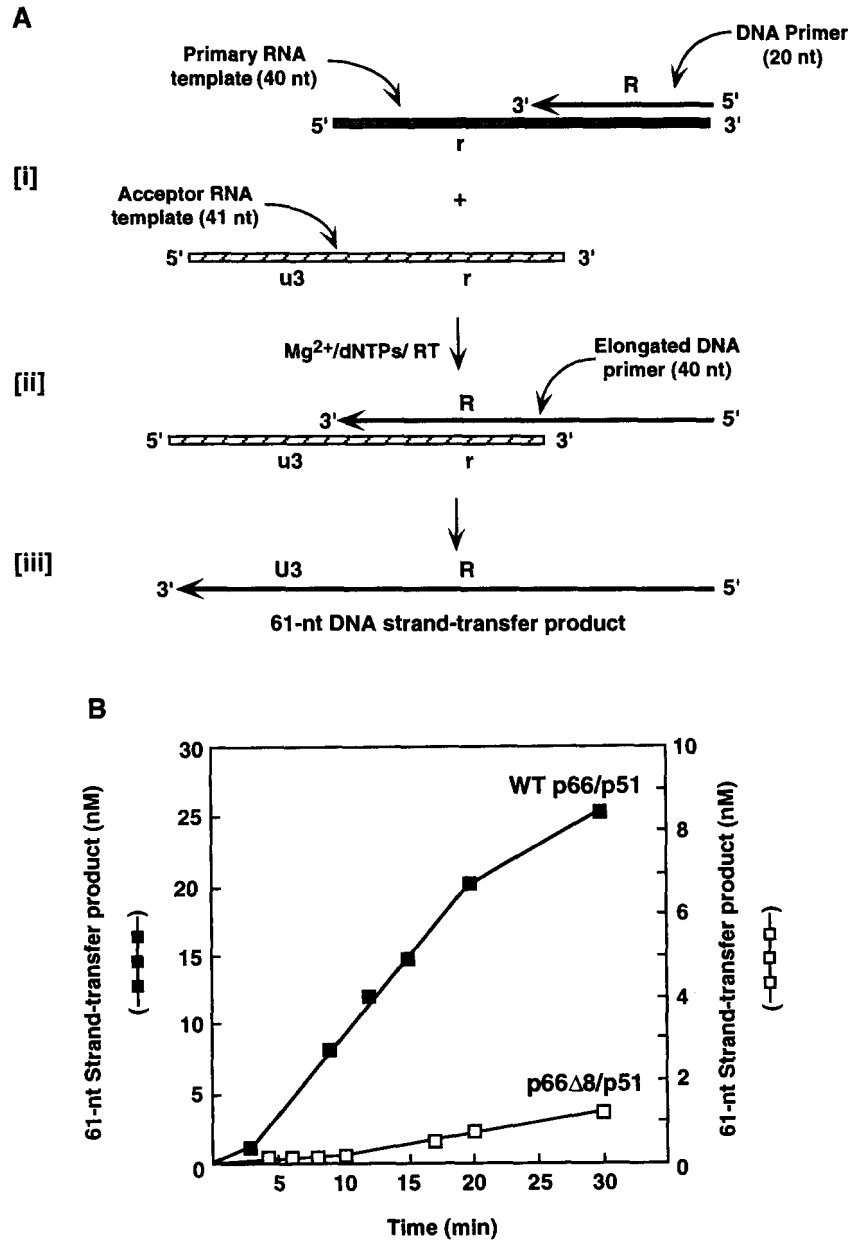

FIG. 4. **Endonuclease activity of p66Δ8/p51 RT fails to support DNA strand transfer.** *A*, features of the strand transfer system. The components of the assay (*i*) comprise a 40-nt donor RNA template derived from the repeat (*r*) end of the HIV-1 genome which is primed with a 20-nt DNA primer and a 41-nt acceptor RNA, derived from the u3 genomic sequence, with homology to the last 20 bases of the donor template. Following primer extension to the 5′ terminus of the donor template (*ii*), a combination of polymerase-dependent and independent RNase H activities allow release and transfer of nascent 40-nt DNA to the acceptor template. Subsequent primer extension on the acceptor template yields a 61-nt strand transfer product. *B*, quantitative analysis of the strand transfer products derived from reactions catalyzed by wild type p66/p51 HIV-1 RT (*closed symbols*) and the deletion mutant p66Δ8/p51 (*open symbols*). Product bands were visualized by phosphorimaging and quantified using ImageQuant software supplied by the manufacturer.

Although DNase I footprinting provides limited resolution, it has recently proven informative in revealing novel features of replication complexes containing variants of murine leukemia virus (39) and HIV-1 RT (40), the latter of which agrees well with structural models for enzyme containing an extended substrate (21). Fig. 5, *A–C*, indicates that subtly altered interactions with the DNA template and primer accompany truncation of the RNase H domain.

In Fig. 5*A*, interaction with the DNA primer of the template-primer duplex (Fig. 1*A*) was evaluated following its extension by a single nucleotide (+1 replication complex). In keeping with earlier data,[5] wild type RT protects as far as position −24 from hydrolysis (*lane 1*). Partial elimination of α-helix E′ in mutant p66Δ8/p51 is accompanied by a moderate increase in DNase I sensitivity at position −23 (*lane 2*). Removal of the entire α helix in mutants p66Δ16/p51 (*lane 3*) and p66Δ23/p51 (*lane 4*) has the consequence that reactivity at positions −24 and −23 is equivalent to that of the naked template-primer duplex. The data of Fig. 5*A* thus illustrate that α-helix E′ of the RNase H domain is sufficiently close to the template-primer duplex to afford protection from DNase I digestion. Fig. 5*B* presents profiles of the template strand in +4 replication complexes. While wild type enzyme protects nucleotides −22 to +6/+7 (*lane 1*), subtle alterations in the template-primer duplex at positions −18/−19 are evident as the size of C-terminal deletion increases (*lanes 2–4*). Limited information is available from these complexes on interactions with the single-stranded portion of the template. This is better illustrated in +10 replication complexes (Fig. 5*C*), where RT advances to protect 6 template nucleotides forming a short hairpin structure near the 5′ extremity of the DNA template (Fig. 1*A*). While the hydrolysis profiles at the leading edge of the replication complexes are identical, reactivity at position −19 again increases with the extent of C-terminal deletion. The combined data of Fig. 5 thus suggest that the manner in which the RNase H domain interacts with the template-primer duplex is influenced by stepwise removal of α-helix E′, while at the same time contacts between the N-terminal p66 fingers subdomain and the single-stranded template are unaffected.

[5] Wöhrl, B. M., Tantillo, C., Arnold, E., and Le Grice, S. F. J. (1995) *Biochemistry*, in press.

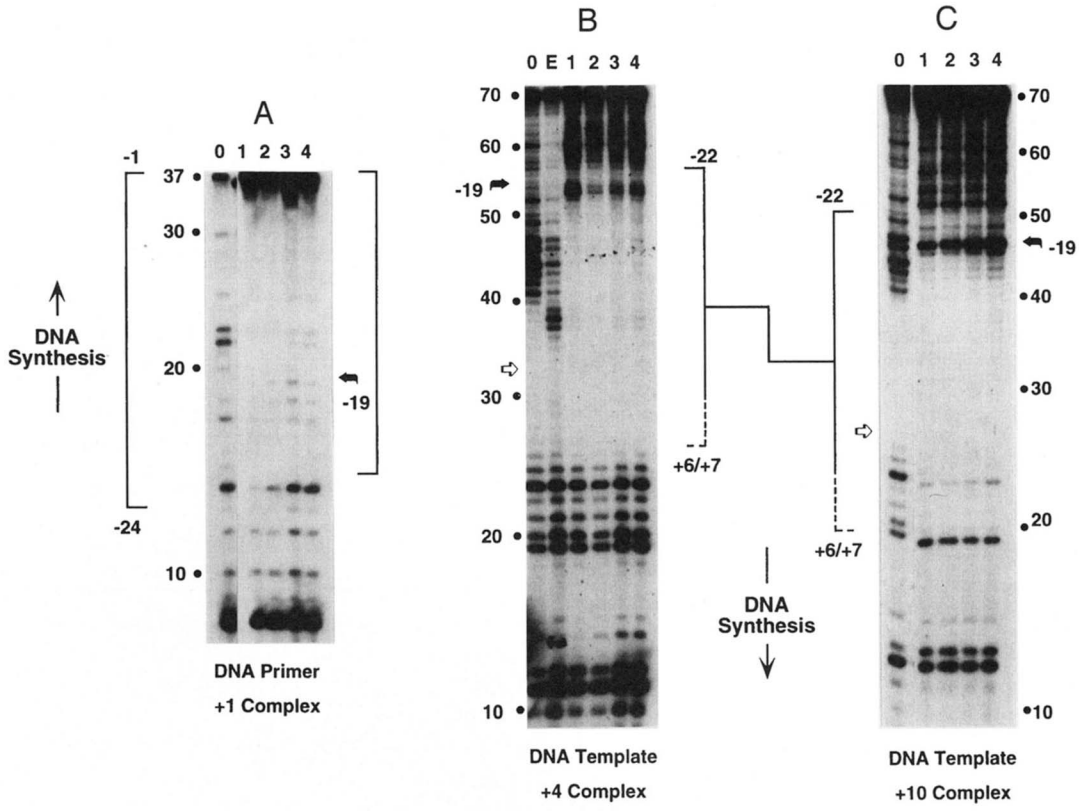

FIG. 5. **DNase I footprinting of replication complexes containing heterodimer mutants.** *A*, protection of primer nucleotides of +1 replication complexes. For these experiments, primer DNA was labeled at its 5′ terminus with ^{32}P. *Lane 0*, control DNase I digest of template-primer in the absence of RT; *lane 1*, wild type p66/p51 RT; *lane 2*, p66Δ8/p51 RT; *lane 3* p66Δ16/p51 RT; *lane 4*, p66Δ23/p51 RT. Protection of primer nucleotide by wild type to position −24 correlates with structural predictions of Nanni *et al.* (21). Positions on the primer where alterations are evident with mutant RT are indicated with *arrows*. *B*, protection of template nucleotides in +4 replication complexes. *Lane 0*, DNase I digest of template-primer duplex. Template DNA was labeled at its 5′ terminus with ^{32}P. *Lane E*, DNase I digest of template DNA on which the primer was extended by 4 nucleotides. *Lane 1*, wild type p66/p51 RT; *lane 2*, p66Δ8/p51 RT; *lane 3*, p66Δ16/p51 RT; *lane 4*, p66Δ23/p51 RT. While the upstream boundary of the replicating enzymes is evident at position −22, interactions with the single-stranded template to position +6/+7 is derived from data of Boyer *et al.* (41). *C*, protection of template nucleotides in +10 replication complexes. *Lane 0*, control digest of template DNA containing an unextended primer. Template DNA was labeled at its 5′ terminus with ^{32}P. *Lane 1*, wild type p66/p51 RT; *lane 2*, p66Δ8/p51 RT; *lane 3*, p66Δ16/p51 RT; *lane 4*, p66Δ23/p51 RT. Alterations to replication complexes containing mutant RT at position −19 are indicated. In *panels B* and *C*, the *open arrow* indicates the position at which the chain-terminating ddNTP was added to the primer (see Fig. 1).

DISCUSSION

Using defined heteropolymeric substrates, we evaluated the consequence of short truncations into the HIV-1 RNase H domain on dimerization, DNA polymerase, RNase H, and strand transfer properties of the parental heterodimer. Although mutagenesis studies on RNase H are available (42–44), these often use *in situ* assays in polyacrylamide gels. In addition to being difficult to quantify, such approaches fail to address the biologically relevant enzyme (the p66/p51 heterodimer) and cannot discriminate between a defective heterodimer or monomers which fail to dimerize, since RT functions are dependent on dimerization (45, 46). Furthermore, the use of defined heteropolymeric substrates should be promoted, since assessing RNase H function as loss of acid-precipitable counts from a randomly-generated hybrid cannot assess whether the endonuclease or directional processing function is selectively impaired. This is well exemplified in a recent study by Post *et al.* (47) with murine leukemia virus RT altered in its connection subdomain or fused to *E. coli* RNase H.

Bearing these caveats in mind, we demonstrate here that removing residues Ser^{553}-Leu^{560} from the RNase H domain of p66 RT yields a stable heterodimer which retains DNA polymerase function, but displays only a subset of its RNase H activities. Loss of directional processing is reflected by low levels of DNA strand transfer, indicating its importance at stages in replication involving strand transfer. Based on data from Peliska and Benkovic (9), we interpret our findings in terms of a mutant capable of copying DNA to the 5′ terminus of the RNA template, after which polymerase-dependent RNase H activity yields a single endonucleolytic cut at or around position −17. Loss of directional processing "locks" RT mutant p66Δ8/p51 with its DNA polymerase active center over the 3′ OH of the fully-extended primer. Should dissociation from template-primer occur, enzyme which rebinds would again be oriented in a manner dictated by the primer terminus (9), thereby preventing template cleavage beyond position −17. p66Δ8/p51 RT is thus "anchored" to the terminus of the template-primer duplex. In the absence of polymerization-independent RNase H activity (9), which we believe is analogous to the directional processing events shown here, the level of strand transfer observed most likely reflects slow melting of the residual 17–18-nt donor RNA fragment from nascent DNA. While the ability to uncouple the two RNase H activities was surprising, support for the dependence of strand transfer on directional processing is provided by studies with the RNase H-deficient enzyme $p66^{E \rightarrow Q}$/p51 (3). In this case, substitution of Mn^{2+} for Mg^{2+} restores endoribonuclease activity, but DNA strand transfer is likewise inhibited.[6]

The crystal structure of the HIV-1 RNase H domain may

[6] N. M. Cirino, C. E. Cameron, J. S. Smith, M. J. Roth, S. J. Benkovic, and S. F. J. Le Grice, submitted for publication.

provide an explanation for our findings. Davies *et al.* (5) propose that four invariant residues, namely Asp^{443}, Glu^{478}, Asp^{498}, and Asp^{549} participate in coordinating two metal ions. Of these, the backbone carbonyl oxygen of Asp^{549} forms a hydrogen bond with the side chain hydroxyl of Ser^{553} of α-helix E′. Since the 8-residue deletion in p66Δ8/p51 eliminates Ser^{553}, reduced constraints on Asp^{549} may have the consequence that binding of one divalent cation is either impaired or eliminated. This notion is not unreasonable, since the model of Davies *et al.* (5) predicts that Asp^{549} and Asp^{443} coordinate one divalent cation (site B), while those of Asp^{443}, Glu^{478}, and Asp^{498} coordinate a second (site A). Thus, if a two-metal ion mechanism of catalysis is correct (5), loss of coordination at site B may influence directional processing. Although the catalytic mechanism for RNase H remains to be established, potential alterations to metal ion coordination cannot significantly influence placement of the RNA-DNA hybrid in the RNase H active site, since cleavage 17–18 nt from the DNA polymerase catalytic center is preserved with mutant p66Δ8/p51. Lack of stalled intermediates during RNA-dependent DNA synthesis by this mutant may indicate that a truncated α-helix E′, coupled with altered metal binding, affords the RNA-DNA hybrid more flexibility following initial endonucleolytic cleavage, thereby rendering directional processing sterically unfavorable.

If structural predictions of Nanni *et al.* (21) are correct, stepwise removal of α-helix E′ of the RNase H domain could alter contacts to the template-primer duplex. Although enzymatic footprinting offers limited resolution, it was capable here of highlighting such alterations (Fig. 5), the most notable being at positions −23 on the primer and −19 on the template. These are rendered DNase-sensitive as the extent of C-terminal deletion increases, supporting the notion that α-helix E′ contributes to the wall of the nucleic acid binding cleft, downstream of the RNase H active center.[2] Although we must interpret DNase I footprinting data with caution, it is interesting to note that interactions of the N-terminal fingers subdomain with template sequences preceding the DNA polymerase active center are unaffected. The importance of these observations lies in a recent communication from Pelletier *et al.* (48), who maintain that during DNA-dependent DNA synthesis, the orientation of RT on template-primer is opposite to that determined by Jacobo-Molina *et al.* (7) and predicted by Kohlstaedt *et al.* (6). According to their model, the RNase H domain occupies the single-stranded template and not the template-primer duplex. This predicts that truncating α-helix E′ should alter protection of the single-stranded template rather than the template-primer duplex. In fact, we observe the opposite. Further proof that their orientation of RT on template-primer is incorrect lies in our analysis of replication complexes containing a variant of murine leukemia virus RT lacking its RNase H domain. While wild type murine leukemia virus RT protects the template-primer duplex to position −27, elimination of the RNase H domain has the consequence that 12 base pairs of the *template-primer duplex* are rendered DNase-susceptible (39). These data provide compelling evidence that during DNA-dependent DNA synthesis, RT is asymmetrically distributed over the primer 3′-OH with the bulk of contact involving the template-primer duplex.

Finally, our data illustrate the importance of qualitative evaluation of HIV RT functions. In the RNase H assays of Fig. 3, both wild type and p66Δ8/p51 RT cleave the RNA-DNA hybrid with equal efficiency, but differ dramatically in their final hydrolysis products. When measuring loss of acid-precipitable counts from a randomly generated RNA-DNA hybrid, differences between these two enzymes would not be immediately apparent (*i.e.* loss of counts would be equal). This predicts that drugs selectively inhibiting directional processing might escape detection, since quantitative evaluation would indicate no alteration to RNase H activity. However, we demonstrate here that loss of this function manifests itself in dramatically reduced DNA strand transfer activity. Implementing qualitative analysis in drug-screening efforts might then highlight novel agents which prevent transfer of DNA between RNA templates during (−)-strand synthesis, exposing stalled replication intermediates to host nucleases.

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