Divalent Cation Modulation of the Ribonuclease Functions of Human Immunodeficiency Virus Reverse Transcriptase†

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ABSTRACT: The stimulatory effect of Mg^{2+} and Mn^{2+} on the ribonuclease H (RNase H) functions of HIV-1 reverse transcriptase (RT) has been evaluated using a model 90-nt RNA template/36-nt DNA primer. Wild type enzyme exhibits similar endonuclease and directional processing activities in response to both cations, while RNase H* activity (hydrolysis of double-stranded RNA) is only evident in the presence of Mn^{2+}. Enzyme altered at the p66 residue Glu478 ($Glu^{478}\rightarrow Gln^{478}$), which participates in metal ion binding, is completely inactive in Mg^{2+}. However, Mn^{2+} restores specifically its endoribonuclease activity. In the presence of Mn^{2+}, mutant RT also catalyzes specific removal of the tRNA replication primer, eliminating the possibility of contaminating *Escherichia coli* RNase H in our recombinant enzyme. However, the efficiency with which mutant RT catalyzes transfer of nascent DNA between RNA templates (an event mandating RNase H activity) is severely reduced. These findings raise the possibility that directional processing activity is required to accelerate transfer of nascent DNA between templates during retroviral replication.

Ribonuclease H (RNase H; RNA−DNA hybrid ribonucleotide hydrolase, EC 3.1.4.34), first documented in 1970 by Hausen and Stein, degrades the RNA component of DNA−RNA hybrids. While a direct role for the bacterial and mammalian enzymes in nucleic acid metabolism remains to be established, work with several retroviruses has been more informative, implicating RT[1] associated RNase H activity at specific stages of the replication cycle (Moelling et al., 1971). These include (a) facilitating transfer of (−) strand strong-stop DNA to the 3′ terminus of the viral RNA genome, (b) generating the (+) strand polypurine-tract primer from the RNA−DNA replication intermediate, and (c) removal of the tRNA and polypurine tract primers. These events [reviewed in Champoux (1993) and Telesnitsky and Goff (1993b)], in coordination with RNA- and DNA-dependent DNA polymerase activities of the same enzyme, yield a double-stranded preintegrative DNA uninterrupted by ribonucleotides. The absolute requirement for virus-associated RNase H function (Schatz et al., 1990b; Tisdale et al., 1991) thus offers several events as targets for antiviral drugs to control spread of human immunodeficiency virus (HIV) infection and the devastating consequences of acquired immunodeficiency syndrome (AIDS).

The attractiveness of retroviral RNase H as a therapeutic target is strengthened by a series of communications indicating that this C-terminal RT domain has an expanded repertoire of catalytic activities. By using heteropolymeric RNA−DNA hybrids whose RNA component was radiolabeled at either the 5′ or 3′ terminus, Schatz *et al.* (1990b) could show that RNase H function was a combination of endonuclease and exonuclease activities (although we currently believe that the term "directional processing" is more accurate than 3′ → 5′-exonuclease), a notion recently confirmed by Zhan *et al.* (1994). The latter studies also proposed that the RNase H domain might exist in conformations favoring the different nuclease functions. In the presence of Mn^{2+}, directional processing activity predominates, while both activities were catalyzed with equal efficiency in the presence of Mg^{2+}. The notion of divalent cation-modulated enzyme function is not unique to the HIV RT, but has also been reported for calf thymus RNase H (Büsen, 1980). A further example of this is phosphorylase kinase, demonstrated by Yuan *et al.* (1993) to phosphorylate serine in the presence of Mg^{2+}, while Mn^{2+} catalyzes phosphorylation of tyrosine. Since Mn^{2+} has been demonstrated to bind at two sites in the HIV-1 RNase H domain (Davies et al., 1991), the manner in which these are occupied could contribute to predominance of a particular nuclease function.

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[1] Abbreviations: HIV, human immunodeficiency virus; IPTG, isopropyl-b-D-thiogalactopyranoside; nt, nucleotides; RNase H, ribonuclease H; RT, reverse transcriptase.

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Recent data with RT from human and murine retroviruses indicates that RNase H activity is not restricted to RNA−DNA hybrids. Initially, Ben-Artzi *et al.* (1992) presented evidence that the RNase H domain of HIV-1 RT was capable of hydrolyzing double-stranded RNA in the presence of Mn^{2+}. Although the biological significance of this activity, now designated RNase H* (12), remains unclear, its general existence was confirmed by a report from Blain and Goff (1993), who showed that Moloney murine leukemia virus (MoMLV) RT-RNase H catalyzed a similar Mn^{2+}-dependent reaction. Since both studies used an *in situ* analysis in polyacrylamide gels (where activity is attributed to renatured proteins by their molecular mass), ambiguity arising from *Escherichia coli* RNase III or RNase HI contamination (Hostomsky et al., 1992) was avoided. Analysis of MoMLV RT mutants also showed that RNase H and RNase H* activities could be uncoupled, suggesting that the C-terminal RT domain might alter its conformation to accommodate hybrid and duplex RNA, which Salazar *et al.* (1993) have demonstrated exist in different conformations. Thus, RNase H* activity might also lend itself to chemotherapy.

The possibility that Mn^{2+} induces a conformation in the RNase H domain favoring a particular activity prompted us to re-evaluate the HIV-1 RT mutant $p66^{E\rightarrow Q}/p51$, which is fully competent as a DNA polymerase but lacks RNase H activity in the presence of Mg^{2+} as well as Mn^{2+}-dependent RNase H* function (Ben-Artzi et al., 1992). Using a defined RNA−DNA hybrid on which static replication complexes could be prepared, we report here that endoribonuclease function of this mutant can be selectively and quantitatively restored by substituting Mn^{2+} for Mg^{2+}. Uncoupling RNase H functions allowed us to evaluate their contribution toward an important event in retroviral replication, namely transfer of nascent DNA within, or between, strands of the retroviral genome.

EXPERIMENTAL PROCEDURES

Enzymes. Wild type p66/p51 HIV-1 RT was prepared from recombinant *E. coli* harboring plasmid pRT6H-PR (Schatz et al., 1989) and the RNase H-deficient mutant from its counterpart, $p6HRT^{E\rightarrow Q}PR$ (Le Grice and Grüninger-Leitch, 1990). Both enzymes were purified to >95% homogeneity by a combination of metal chelate and ion exchange chromatography (Le Grice et al., 1991). By using poly r(A)/oligo$(dT)_{12-18}$ as template/primer, a specific RNA-dependent DNA polymerase activity of 30−40 units/mg was determined for each enzyme, where 1 unit catalyzes incorporation of 1 nmol precursor dTTP into polydeoxynucleotide in 10 min at 37 °C.

Qualitative Assessment of DNA Polymerase and RNase H Function. (a) DNA-Dependent DNA Synthesis. DNA-dependent DNA synthesis was determined on a heteropolymeric 71-nt/36-nt template/primer according to Wöhrl et al. (1994) and Ghosh et al. (1995). Assay mixtures (20 μL) contained 100 mM HEPES, pH 7.8, 6 mM $MgCl_2$ or $MnCl_2$, 80 mM KCl, 1 mM DTT, 0.5 pmol of radiolabeled template/primer, and 0.5−1.0 pmol of HIV-1 RT. The enzyme was incubated with template/primer for 1 min at 37 °C, after which polymerization was initiated by addition of dNTP/ddNTP mixtures (final concentration 50 μM dNTP, 500 μM ddNTP) permitting primer extension by 4, 10, or 36 nucleotides. Synthesis was terminated after 10 min by adding an equal volume of a urea-containing gel loading buffer (7 M urea in Tris/borate/EDTA buffer containing 0.1% xylene cyanol and bromophenol blue). Polymerization products were fractionated by high-resolution gel electrophoresis through 10% polyacrylamide gels containing 7 M urea and analyzed by autoradiography.

(b) RNA-Dependent DNA Synthesis. The 71-nt DNA template of the previous section was copied with DNA polymerase Klenow fragment and inserted in the correct orientation into a commercially-available plasmid immediately downstream of a bacteriophage T7 promoter. Cleavage of this plasmid (p90RNATem) with *Sma*I, followed by *in vitro* transcription with T7 RNA polymerase (Ambion, TX), yielded a 90-nt template which differed from its DNA counterpart in that the 5′ terminus was extended by 19 nucleotides (derived from polylinker sequences in the plasmid). When hybridized to the radiolabeled 36-nt DNA primer and incubated with HIV RT under the conditions described in (a), analogous "programmed" primer extension was achieved, with the exception that the full-length cDNA product was 90 nt.

(c) RNase H Activity. The 90-nt RNA template/36-nt DNA primer of (b) was used as substrate for analysis of RNase H function by relocating radiolabel to the template 5′ terminus. RNase H activity was determined following addition of 1, 4, or 10 nucleotides to the primer (+1, +4, and +10 complexes, respectively). RNase H hydrolysis products were fractionated and analyzed as described above.

tRNA Primer Removal. The specificity with which each RT excised the tRNA replication primer from an RNA/DNA chimera was determined as described by Smith and Roth (1992), with the following modifications. Twenty pmol of 18-mer HPR-1 RNA primer (5′-GUCCCUGUUCGGGCGCCA-3′) were labeled at the 5′ terminus with γ-[^{32}P] ATP and polynucleotide kinase, after which 10 pmol were hybridized to 40 pmol of 38-mer HTD-1 DNA (5′-GTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGAC-3′). Hybridized 18-mer RNA was extended with the exonuclease (−) Klenow fragment of DNA polymerase I (USB) in a 20 μL reaction mixture containing 23 mM Tris−HCl, pH 7.4, 5 mM $MgCl_2$, 35 mM NaCl, 1.5 mM DTT, 0.4 mM dCTP, TTP, dATP, and dGTP. The RNA−DNA chimera was purified by denaturing electrophoresis through a 15% polyacrylamide gel. The substrate for RNase H assays was prepared by annealing 8 pmol of gel-purified 38-mer RNA−DNA chimera to 20 pmol of HTD-1 38-mer DNA.

tRNA primer removal assays were conducted at 37 °C in a buffer of 20 mM Tris−HCl, pH 7.5, 50 mM KCl, 8 mM $MgCl_2$ or $MnCl_2$, 2 mM DTT, and 0.4 pmol of radiolabeled substrate. Reactions were initiated by adding 1.0 pmol of wild type p66/p51 RT or the RNase H-deficient mutant. As controls, equivalent reactions were carried out with p51 HIV-1 RT (1.0 pmol) or 0.004 units of *E. coli* RNase H (= 0.25 fmol). Aliquots were removed at several intervals and mixed with a solution of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. Reaction products were fractionated by high voltage electrophoresis through 20% polyacrylamide/7 M urea gels and analyzed by autoradiography.

DNA Strand Transfer Reactions. The efficiency with which wild type and mutant HIV-1 RT catalyzed transfer of nascent cDNA to an RNA acceptor template was determined according to Peliska and Benkovic (1992). Oligonucleitide sequences for these experiments were as follows: Strand transfer reactions were performed in a buffer of 50 mM Tris−

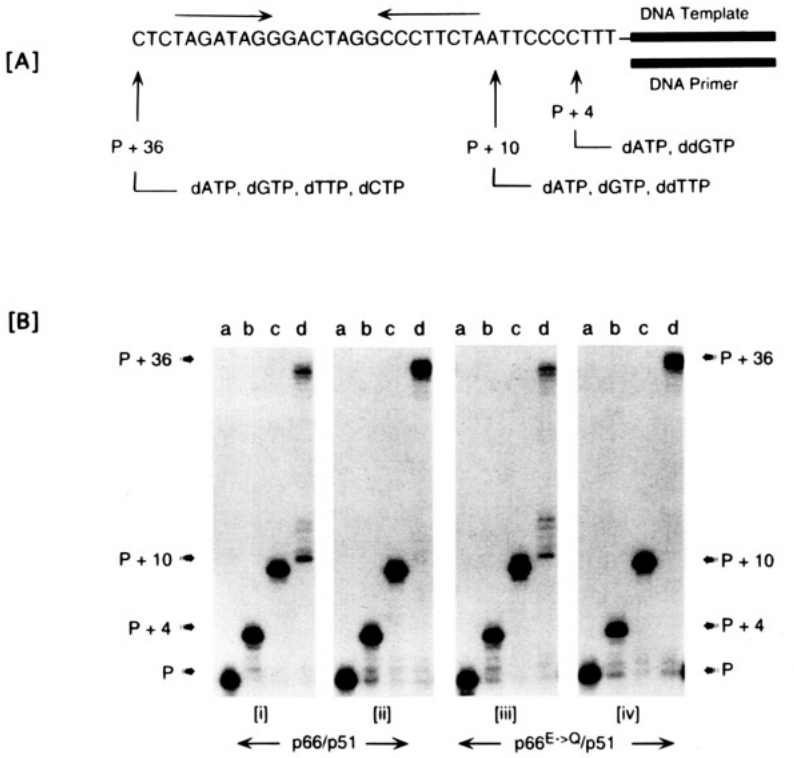

FIGURE 1: Mn^{2+}- and Mg^{2+}-stimulated DNA-dependent DNA polymerase activity of wild type and RNase H-deficient p66/p51 HIV-1 RT. (A) Model template/primer and dNTP/ddNTP combinations for specific primer extension. The shaded portion of the substrate is a 35 bp template/primer duplex (Wöhrl et al., 1994, 1995). Inverted repeat sequences giving rise to a hairpin structure on the single-stranded template are indicated by arrows. (B) Mn^{2+}- (panels i and iii) and Mg^{2+}-catalyzed (panels ii and iv) reactions. In these experiments, the DNA primer was labeled at its 5′ terminus with [^{32}P]. Lane: (a) unextended primer (P); (b) +4 extension reaction (P + 4); c, +10 extension reaction (P + 10); d, complete primer extension reaction (P + 36).

(i) 5′-AGAGCTCCCAGGCTCAGATC-3′

(20-nt DNA primer)

(ii) 3′-UCUCGAGGGUCCGAGUCUAGACU
AGAUUGGUCUCUCUGGG-5′

(40-nt donor RNA template)

(iii) 3′-ACCAGAUUGGUCUCUCUGGGU
CAUGUCCGUUUUUCGUCGAG-5′

(41-nt acceptor RNA template)

HCl, pH 8.0, 75 mM KCl, 1 mM DTT, 0.1% Triton X-100, 7 mM $MgCl_2$ or $MnCl_2$, 100 μM dATP, dGTP, dCTP, dTTP, 200 nM 5′ end-labeled 20-nt DNA/40-nt RNA, 480 nM acceptor RNA template, and 200 nM HIV-1 RT. Reactions were initiated at 37 °C by addition of RT; at the times indicated in the text, samples were withdrawn and DNA synthesis terminated by addition of EDTA to a final concentration of 110 mM. Products were fractionated by high voltage electrophoresis through 15% polyacrylamide gels containing 7 M urea, visualized with a Molecular Dynamics phosphorimager and quantified using ImageQuant software provided by the supplier.

RESULTS

Mg^{2+} and Mn^{2+} Dependent DNA Polymerase Activity. We (Wöhrl et al., 1994; Jacques et al., 1994) and others (Telesnitsky and Goff, 1993a) have noted that retroviral RTs with equivalent specific RNA- or DNA-dependent DNA polymerase activity can differ significantly in processivity of DNA synthesis. In a preliminary analysis, we therefore determined the efficiency with which $p66^{E\rightarrow Q}$/p51 and wild type RT extended the primer of related DNA−DNA and RNA−DNA template−primer combinations in response to the divalent cation activator. The results of this analysis are presented in Figures 1 and 2.

In Mg^{2+}, wild type RT and mutant $p66^{E\rightarrow Q}$/p51 quantitatively catalyze 4- and 10-base primer extension on a DNA template and traverse this unimpaired in the absence of a chain-terminating ddNTP (Figure 1B, panels ii and iv). The same figure illustrates that substituting Mn^{2+} for Mg^{2+} minimally influences the efficiency of the 4- and 10-base primer extension reactions (panels i and iii). However, in the absence of chain termination, Mn^{2+}-dependent polymerization products of both enzymes partition between fully-extended primer and a subpopulation indicative of stalling 11−15 bases along the template (Figure 1B, panels i and iii). This corresponds to a position on the DNA template where a hairpin structure is assumed (Figure 1A; Wöhrl et al., 1995). Since both enzymes traverse the template up to and beyond this hairpin with equal efficiency, Mn^{2+} does not appear to influence the polymerizing enzyme *per se*, but may rather stabilize the hairpin. Although minor differences in Mn^{2+}-catalyzed DNA synthesis can be detected, both wild type and mutant RT respond similarly. The same observation was made when DNA-dependent DNA polymerase activity was assessed in the presence of the competitor heparin, which restricts each enzyme to a single cycle of synthesis (data not shown).

Figure 2 illustrates RNA-dependent DNA polymerase activity on a closely-related RNA template (differing at its

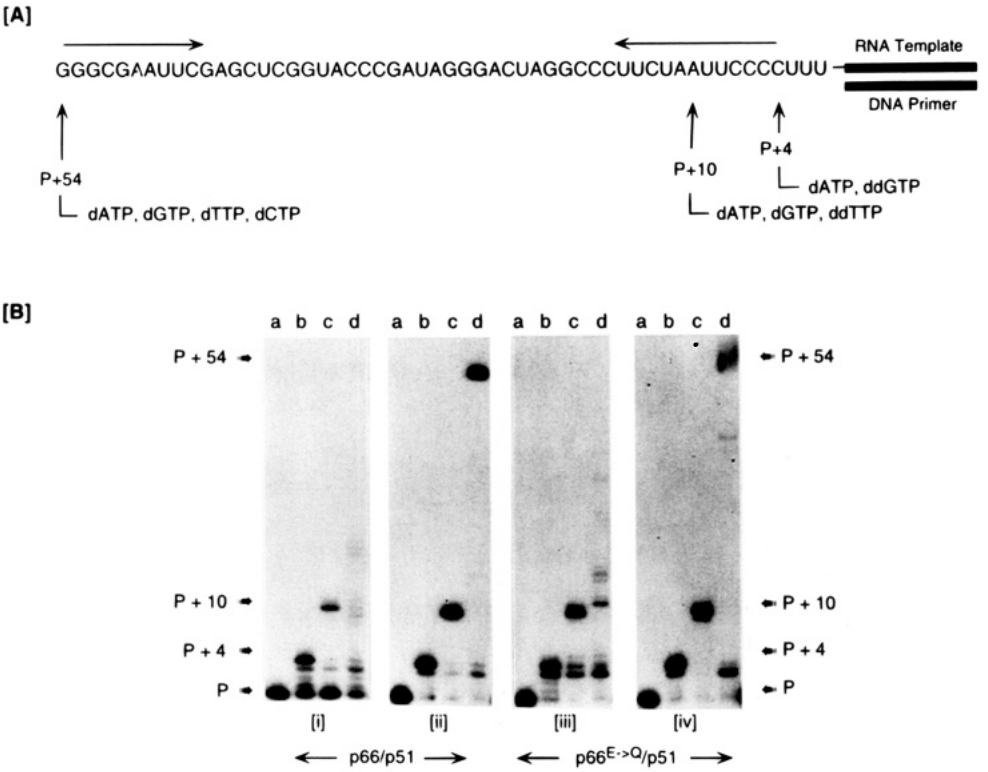

FIGURE 2: Mn^{2+}- and Mg^{2+}-stimulated RNA-dependent DNA polymerase activity of wild type and RNase H-deficient RT. (A) 90-nt RNA template/36-nt DNA primer. The template/primer duplex and adjacent 31 template nucleotides are analogous to those of Figure 1, while an additional 19 nucleotides are vector-derived. Conditions for specific primer extension reactions are illustrated. Inverted repeat sequences on the single-stranded template are indicated by arrows. (B) Mn^{2+}- (panels i and iii) and Mg^{2+}-catalyzed (panels ii and iv) reactions. Lane notations are similar to those of Figure 1.

5′ terminus; Figure 2A). In the presence of Mg^{2+}, both enzymes extend the DNA primer as expected, with the exception that minor stalling occurs at positions P + 3 and P + 4 (Figure 2B, panels ii and iv). However, substitution with Mn^{2+} has more significant consequences for RNA-dependent DNA synthesis. Although wild type RT supports 4- and 10-base extension reactions, pausing is evident at position P + 3, and a considerable amount of unextended primer remains. Furthermore, in the absence of a chain terminator, stalled products predominate and little fully-extended primer (P + 54) is synthesized (Figure 2B, panel i). When the same conditions are applied to $p66^{E\rightarrow Q}/p51$ RT, subtle differences in processivity of DNA synthesis were apparent. The absence of unextended primer suggests that the rate of DNA synthesis is increased in the presence of Mn^{2+} (Figure 2B, panel iii). However, the same enzymes appear to be more severely stalled at position P + 3. In a similar manner to its wild type counterpart, mutant RT supports minimal full-length cDNA synthesis in Mn^{2+}, while the distribution of stalled intermediates is slightly different.

Accumulation of stalled intermediates during RNA-dependent DNA synthesis suggested that the single-stranded RNA template may also adopt an intramolecular base-pairing. Computer-assisted folding programs and partial hydrolysis of the RNA template with RNase A (hydrolyzes at pyrimidne residues of single-stranded RNA), RNase CV (hydrolyzes double-stranded RNA), and nuclease S1 (hydrolyzes single-stranded nucleic acid) both suggest a hairpin structure where the template 5′ terminus hybridizes to sequences in the immediate vicinity of the template/primer duplex (Figure 2A; N. M. Cirino and S. Le Grice, unpublished observations). If correct, this would account for stalling of RT at positions P + 3 and P + 4.

Mn^{2+} Restores Specifically Endoribonuclease Activity to Mutant $p66^{E\rightarrow Q}/p51$. Relocating radiolabel to the 5′ terminus of the RNA template allowed us to use the RNA/DNA hybrid of Figure 2A for a similar evaluation of RNase H activity. On the basis of data from Peliska and Benkovic (1992) and DeStefano *et al.* (1993), indicating that HIV-1 RT positions itself about the primer 3′ terminus, we monitored RNase H activity following three different synthetic events, thereby co-ordinating RNase H and DNA polymerase activities. The results of this analysis are presented in Figure 3.

Figure 3A, panel ii, demonstrates that during DNA synthesis, wild type RT hydrolyzes the RNA template in Mg^{2+} in a manner illustrative of its endonuclease and directional processing activities (Schatz et al., 1990; Zhan et al., 1994). Following extension of the primer by a single base, the 71-nt and 62-nt hydrolysis products (relative to radiolabel at the template 5′ terminus) define the boundaries of RNase H activity. For RT whose DNA polymerase catalytic center is positioned over the 3′ OH of the primer, the 71-nt product predicts hydrolysis at position −18, agreeing with estimates of the distance between the DNA polymerase and RNase H catalytic centers (Wöhrl and Moelling, 1990; DeStefano et al., 1991; Furfine and Reardon, 1991a,b; Gopalakrishnan et al., 1992; Fu & Taylor, 1992). The 62-nt hydrolysis product corresponds to cleavage at position −9, which is proximal to the DNA polymerase catalytic center defined by X-ray crystallography (Jacobo-Molina et al., 1993) and chemical footprinting (Metzger et al., 1993). A similar cleavage pattern has been observed by others (Furfine and Reardon, 1991a,b), suggesting that initial endonucleolytic cleavage is accompanied by directional processing to position −9.

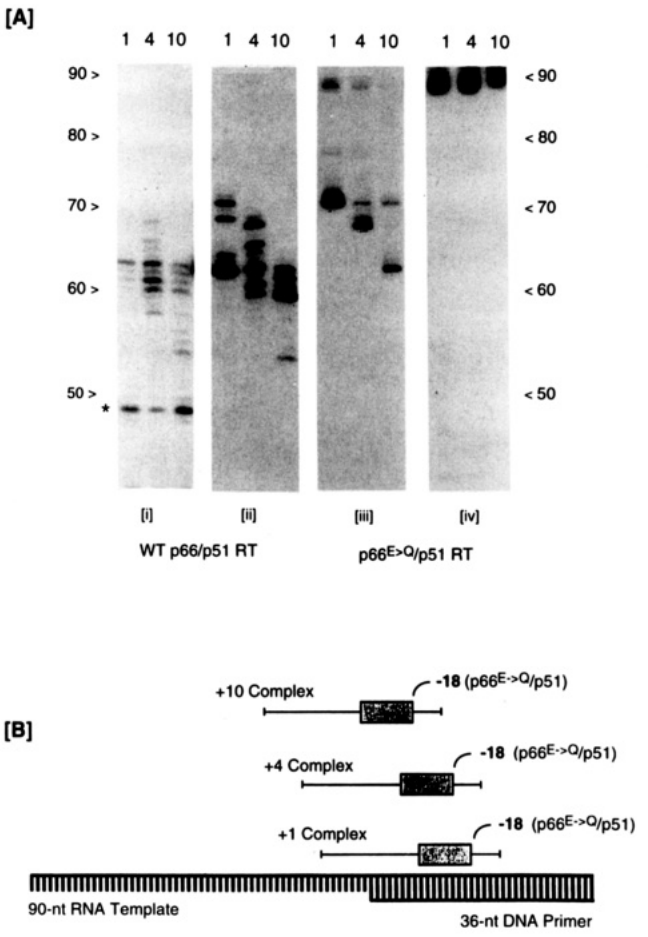

FIGURE 3: Mn^{2+}-dependent endoribonuclease activity of $p66^{E\rightarrow Q}$/p51 HIV-1 RT. (A) RNase H activity of wild type (panels i and ii) and mutant RT (panels iii and iv) following limited DNA synthesis. The 90-nt RNAtemplate/36-nt DNA primer of Figure 2A was used to determine RNase H activity by relocating radiolabel to the template 5′ terminus. Notations above each panel represent nucleotides added to the DNA primer. Nucleotide marker lengths were derived from partial RNase A and alkaline hydrolysis profiles of the template. The migration position of uncleaved RNA template is represented by the 90-nt molecular weight marker. In panel i, * indicates RNase H* cleavage of the template. (B) Schematic representation of the RNase H hydrolysis profiles of wild type RT and mutant $p66^{E\rightarrow Q}$/p51. For each complex, template nucleotides covered by the replicating enzyme are enclosed within the bars (Wöhrl et al., 1995). The limits of RNase H cleavage by wild type RT (in Mg^{2+} and Mn^{2+}) are indicated by the shaded box. In contrast, mutant RT cleaves only at position −18 of the three replication complexes in Mn^{2+}.

A 4-nt primer extension (Figure 2) is accompanied by an initial endonucleolytic cleavage 18 nucleotides behind the new primer terminus (68 nt product; Figure 3) and directional processing to position −9 (59-nt product; Figure 3). Intermediate species are most likely the consequence of stalling, as evidenced in the data of Figure 2B. Finally, addition of 10 nucleotides to the primer is accompanied by accumulation of RNase H hydrolysis products between 62 and 53 nt, i.e., 18 and 9 nt behind the +10 primer terminus. In the presence of Mn^{2+}, the same RNase H cleavage products are generated by wild type RT, although this analysis is complicated by simultaneous induction of RNase H* activity (11) on duplex structures assumed by the RNA template (Figure 3A, panel i).

In keeping with our previous studies (Schatz et al., 1989; Ben-Artzi et al., 1992), RNase H and RNase H* activities are absent from heterodimer $p66^{E\rightarrow Q}$/p51 RT in the presence of Mg^{2+} (Figure 3A, panel iv). Surprisingly, when we substituted Mn^{2+} for Mg^{2+}, mutant RT cleaved the RNA template of all replication complexes (Figure 3A, panel iii). The complete absence of RNase H cleavage products in Mg^{2+}-catalyzed reactions (panel iv) and the manner in which Mn^{2+}-dependent cleavage products are distributed relative to the translocating enzyme (panel iii) rule out the possibility of *E. coli* RNase H contamination. However, when compared to the hydrolysis profiles generated by wild type RT, mutant $p66^{E\rightarrow Q}$/p51 appears to recover only a subset of its RNase H activities in Mn^{2+}. In a +1 replication complex, the 71/72-nt cleavage products correspond to positions −18/−19. Extending the DNA primer by 4 and 10 nucleotides results in predominant cleavage at position −18 of the respective replication complex. Thus, although Mn^{2+} restores RNase H activity to RT mutant $p66^{E\rightarrow Q}$/p51, the data of Figure 3A (panel iii) imply that this is restricted to endoribonuclease function.

Mutant RT Cleaves a Substrate Mimicking tRNA Primer Removal in the Presence of Mn^{2+}. Using synthetic RNA−DNA chimeras, Furfine and Reardon (1991a,b) and Smith and Roth (1992) constructed model substrates to evaluate RNase H cleavage of a replication intermediate mimicking the step at which $tRNA^{Lys,3}$ is released from nascent (−) strand DNA (Figure 4A). RT mutant $p66^{E\rightarrow Q}$/p51 was therefore evaluated for its capacity to cleave specifically at the RNA−DNA junction of this substrate as a function of the divalent cation activator. A valuable feature of the system is the difference in hydrolysis profiles generated by bacterial and retroviral RNase H, which is a sensitive probe for contamination by the bacterial enzyme. The results of this experiment are presented in Figure 4B.

In Mg^{2+}, wild type p66/p51 RT initially cleaves the RNA−DNA chimera endonucleolytically to release a 17-nt RNA (Figure 4B, panel i). This corresponds to hydrolysis at the penultimate phosphodiester bond of the 18-mer oligoribonucleotide as observed by Smith and Roth (1992). Following prolonged incubation, the primary hydrolysis product is shortened to 8 nt, where it presumably dissociates from the complementary DNA strand. Wild type RT thus contains high levels of directional activity and behaves as predicted. Under the same conditions, RNase H activity is completely absent from RT mutant $p66^{E\rightarrow Q}$/p51 (Figure 4B, panel ii). Mg^{2+}-catalyzed cleavage of the RNA−DNA chimera by *E. coli* RNase H reveals a strikingly different hydrolysis profile, indicative of random cleavage (panel iii). Comparison of Figure 4B (panels ii and iii) demonstrates unambiguously that our RNase H-deficient HIV enzyme is free of contaminating *E. coli* RNase H.

Replacing Mg^{2+} with Mn^{2+} has little consequence for the manner in which wild type RT cleaves the RNA−DNA chimera, with the exception that the initial 17-nt hydrolysis product is more rapidly cleaved (Figure 4B, panel iv). This would imply that in Mn^{2+}, directional processing activity is enhanced, which agrees with recent data of Zhan *et al.* (7). At the same time, no *E. coli* RNase H activity is detected (panel vi), despite incubation for 30 min. However, the specific 17-nt hydrolysis product is evident when the chimeric substrate is incubated with $p66^{E\rightarrow Q}$/p51 RT (panel iv). Although considerably less 17-nt product is generated, endonucleolytic cleavage specificity appears to have been preserved. Prolonged autoradiographic exposure fails to reveal smaller cleavage products, indicating loss of directional processing activity. The data of Figures 3 and 4 are

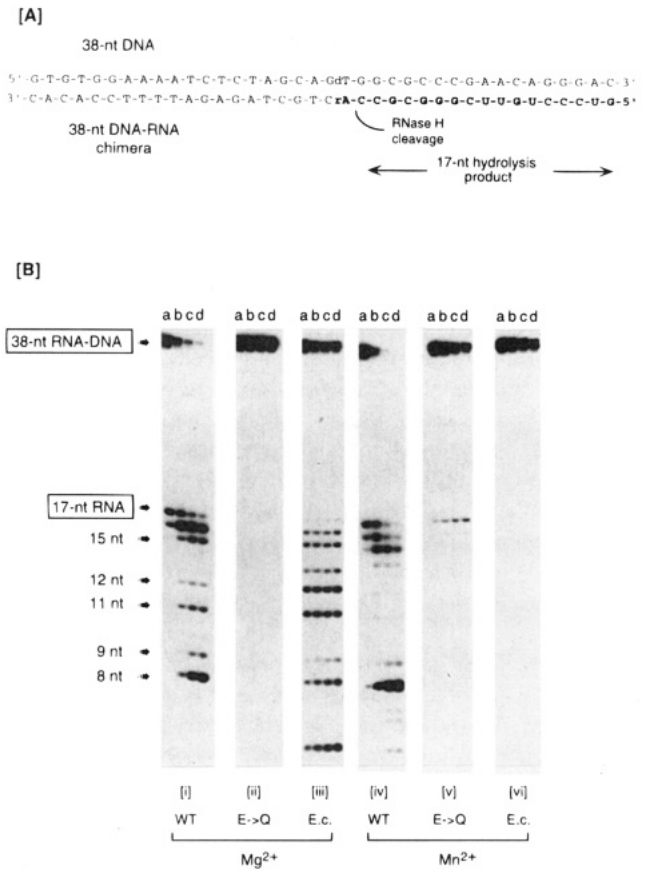

FIGURE 4: Efficiency of tRNA primer removal by wild type and mutant p66/p51 HIV-1 RT. (A) Chimeric DNA−RNA substrate. The upper strand of the duplex is a 30-nt DNA oligonucleotide, while the lower, chimeric strand contains 18 ribonucleotides representing the 3′ acceptor stem of $tRNA^{Lys,3}$. Note that the initial RNase H cleavage occurs at the penultimate ribonucleotide (Smith and Roth, 1992). (B) Mg^{2+}- (panels i−iii) and Mn^{2+}-catalyzed (panels iv−vi) tRNA primer removal. For comparison, *E. coli* RNase H (E.c.) was included. For panels ii, iii, v, and vi, lanes a−d represent RNase H activity following incubation for 2, 5, 15, and 30 min, respectively. In Panels i and ii, incubation times were 10 s (a), 2 min (b), 15 min (c), and 30 min (d). The migration position of the uncleaved RNA−DNA chimera and the primary RNase H cleavage product are indicated in boxes at the left panel (i). Nucleotide lengths were determined from radiolabeled oligonucleotide markers.

thus consistent in that (a) contaminating *E. coli* RNase H does not account for the activity of mutant $p66^{E\rightarrow Q}$/p51 and (b) while RNase H activity can be restored by altering the divalent cation requirement, this is restricted to endoribonuclease function.

Endoribonuclease Activity Alone Fails To Support DNA Strand Transfer. Peliska and Benkovic (1992) have implicated "polymerase-dependent" and "polymerase-independent" modes of RNase H activity during DNA strand transfer, an obligatory step in the retroviral replication cycle (Telesnitsky and Goff, 1993). In the first of these, extension of nascent (−) strand DNA to the 5′ terminus of the RNA template is accompanied by RNase H-mediated cleavage at position −17 (relative to the primer terminus). Polymerase-independent RNase H activity (of the same or another molecule) subsequently cleaves from this position toward the template 5′ terminus. Transfer of nascent DNA initiates when the RNA template within the replication complex is reduced to ~8 nt, presumably reflecting its thermal instability and dissociation. These two mechanisms can be likened to the endonuclease and directional processing activities we have demonstrated for p66/p51 HIV-1 RT (Schatz et al., 1990a,b). If so, loss of the latter function in $p66^{E\rightarrow Q}$/p51 might have a direct consequence for DNA strand transfer. This notion was borne out by the data of Figure 5.

The features of our model DNA strand-transfer system (Figure 5A) are similar to those described by Peliska and Benkovic (1992). Extension of a 20-nt DNA primer to the 5′ terminus of the donor RNA template yields a 40-nt strand transfer intermediate. Transfer to the acceptor template and resumption of DNA synthesis yields a 61-nt product. Accumulation of these DNAs is illustrated in Figure 5B,C. Wild type RT catalyzes a rapid burst in synthesis of the 40-nt transfer intermediate, the amount of which declines after ~10 min (Figure 5B), concomitant with which is a rise in the 61-nt strand transfer product. The data of Figure 4B indicate that Mn^{2+} can substitute for Mg^{2+} without significantly influencing the efficiency of strand transfer. Although the kinetics of DNA synthesis are different with mutant $p66^{E\rightarrow Q}$/p51, significant amounts of the 40-nt strand transfer intermediate accumulate in both Mg^{2+}- and Mn^{2+}-catalyzed reactions (Figure 5C). The absence of a 61-nt strand transfer product in Mg^{2+}-catalyzed reactions is explained by total loss of Mg^{2+}-dependent RNase H function. However, an equivalent finding in the presence of Mn^{2+} demonstrates that restoring endoribonuclease function of mutant $p66^{E\rightarrow Q}$/p51 (Figures 3 and 4) is clearly insufficient to support DNA strand transfer. The data of Figure 5 thus imply that directional processing activity RT is invoked during replication at the stage where the donor RNA template must be reduced to a size favoring strand transfer.

DISCUSSION

The observation that substituting Mn^{2+} for Mg^{2+} is accompanied by relaxed substrate specificity of several restriction endonucleases (Roberts and Halford, 1993) can also be applied to retroviral RT. In addition to their classical role of RNase H activity in degrading RNA/DNA hybrids, the MLV and HIV enzymes will hydrolyze duplex RNA in the presence of Mn^{2+} (Ben-Artzi et al., 1992; Blain and Goff, 1993), although the physiological role of this activity must be established. Furthermore, Zhan et al. (1994) have demonstrated that the endonuclease and directional processing activities of HIV-1 RT can be modulated by altering the divalent cation activator, suggesting different metal ion-induced conformational states. In this paper, we show that Mn^{2+} restores specifically endoribonuclease function to an RT mutant previously shown to lack Mg^{2+}-dependent activity by mutating an invariant residue of the catalytic triad (Glu^{478}). When evaluating RNase H activity of recombinant retroviral RT, low level contamination with bacterial RNase H and RNase III can yield misleading results (Ben-Artzi et al., 1992; Hostomsky et al., 1992). However, the observation here that model RNA/DNA hybrids or chimeras (i) remain intact after incubation with mutant $p66^{E\rightarrow Q}$/p51 for 30 min in Mg^{2+} (Figure 3B) and (ii) are resistant to cleavage by *E. coli* RNase H in Mn^{2+} (Figure 4B) provides compelling evidence that we are monitoring RT-associated activity.

These conditions for selective expression of RNase H function have been exploited to demonstrate that despite retention of DNA synthesis and endoribonuclease activity, mutant $p66^{E\rightarrow Q}$/p51 supports little to no DNA strand transfer. Since the Mn^{2+} concentrations used here (6 mM) cannot be achieved *in vivo* (where it rarely exceeds 0.5 mM), it might

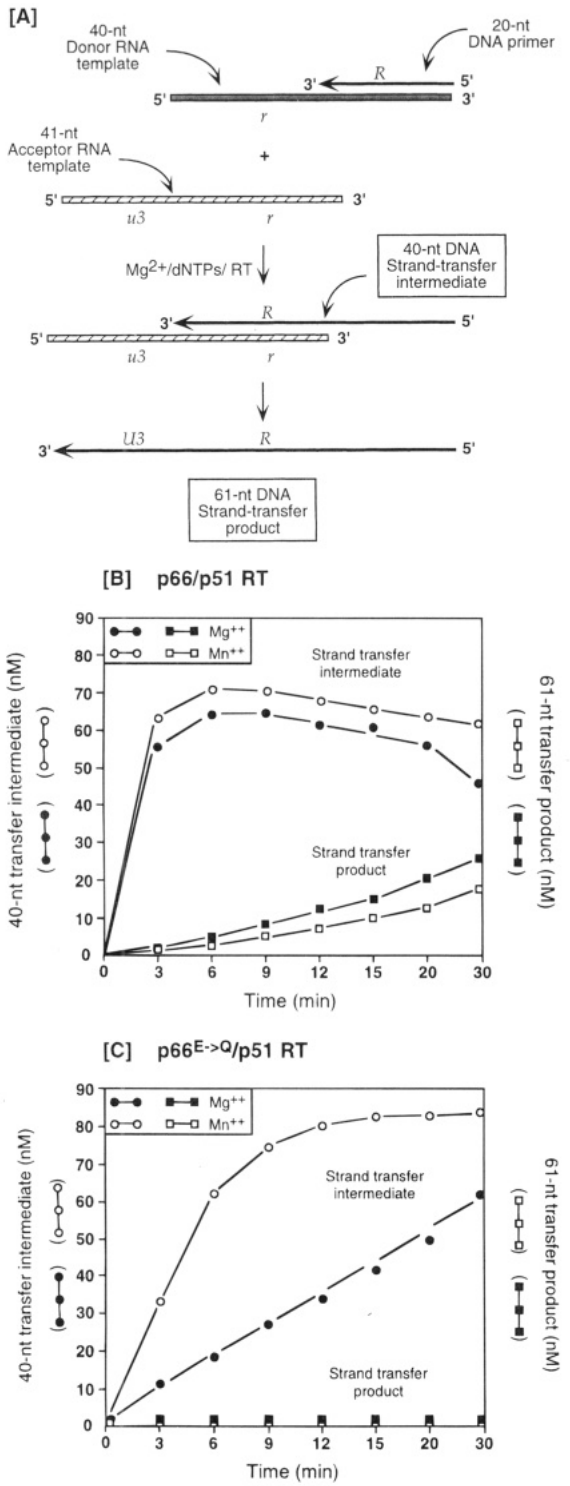

FIGURE 5: Endoribonuclease activity of p66$^{E\rightarrow Q}$/p51 RT fails to support DNA strand transfer. (A) Schematic representation of the DNA strand transfer process. A 40-nt donor RNA template, derived from the repeat (r) end of the HIV-1 genome, is primed with a 20-nt DNA primer. Following extension to the 5′ terminus of the donor, polymerase-dependent and -independent RNase H activities allow release and transfer of nascent 40 nt DNA to an acceptor, 41-nt RNA template. The acceptor is derived from the u3 genomic sequence with homology to the last 20 bases of the donor template. Subsequent primer extension on the acceptor template yields a 61 nt strand transfer product. (B,C) Efficiency of DNA strand transfer catalyzed by wild type p66/p51 (panel B) and mutant p66$^{E\rightarrow Q}$/p51 (panel C). In both cases, open symbols represent activity determined in Mn^{2+}, while closed symbols represent activity in Mg^{2+}. Circles represent accumulation of the 40-nt DNA strand transfer intermediate and squares the 61 nt strand transfer product. DNA products were visualized by phosphorimaging and quantified using ImageQuant software.

be argued that our data are artifactual. However, in a related study, we have prepared a p66/p51 HIV-1 RT mutant whose p66 component lacks eight C-terminal residues, truncating α-helix E′ of the RNase H domain (Jacobo-Molina et al., 1992). In the presence of Mg^{2+}, this mutant (p66Δ8/p51) behaves similarly to p66$^{E\rightarrow Q}$/p51 RT in Mn^{2+}; i.e., although full polymerase and endoribonuclease activity are retained, DNA strand transfer is seriously impaired (Ghosh et al., 1995). Although endonucleolytic and directional processing functions for the HIV-1 RNase H domain have been documented (Schatz et al., 1990b; Zhan et al., 1994), the necessity for *both* during replication was unclear. However, in light of studies of Peliska and Benkovic (1992), a role of directional processing or directional nuclease activity (DeStefano *et al.*, 1991, 1993) in reducing the donor RNA template to a size permitting transfer of nascent DNA can be considered. In the absence of directional processing, our data imply that polymerization to the 5′ terminus of the donor template would "lock" the RNase H domain 17−18 bp behind the primer terminus, following the last endonucleolytic cleavage of the templates. At this stage, two options are open to RT, namely (a) it remains bound in a "stalled" complex or (b) it dissociates and rebinds. In the even of dissociation, the notion of Peliska and Benkovic (1992) that the DNA 3′ OH dictates the orientation of RT during RNA-dependent DNA synthesis predicts that enzyme would rebind in the same manner, i.e., with its RNase H domain still ~18 bp from the primer terminus. Either scenario has the consequence that further hydrolysis of the template cannot be achieved. Consequently, nascent (−) strand DNA will not be released and strand transfer is interrupted. The implication of these events is therefore that directional processing activity of HIV RT is required to complete transfer of nascent DNA within, or between, strands of the retroviral genome.

The crystal structure of the isolated HIV-1 RNase H domain (Davies et al., 1991), contains two Mn^{2+} ions. Metal at site A appears to be co-ordinated to side-chain carboxylates of Glu^{479}, Asp^{498}, and Asp^{443}, while Asp^{443} and Asp^{549} bind metal at site B. This configuration correlates well with the disposition of metal ions in the Klenow fragment of DNA polymerase, from which a two-metal ion catalytic mechanism has been inferred for HIV-1 RNase H (Davies et al., 1991). However, this contrasts with data from Katayanagi *et al.* (1992), whose structure for *E. coli* RNase H at 2.8 Å resolution indicates single metal ion-catalyzed hydrolysis. The latter predicts that the scissile phosphate backbone is positioned for hydrolysis by the side chains of Asp^{10} and Glu^{48}, as well as the main chain carbonyl group of Gly^{11}. This can be accommodated by the HIV-1 RNase H domain, where the counterparts are Asp^{443}, Gly^{444}, and Asp^{478}. Substitution of Mn^{2+} for Mg^{2+} does not alter the specificity of wild type HIV-1 RNase H (Figures 3 and 4), implying that Mn^{2+} is co-ordinated in a similar fashion. However, it does not explain recovery of endonuclease specificity by enzyme mutated at Glu^{478} while directional processing activity is lost. Furthermore, single metal ion-catalyzed hydrolysis does not explain loss of exonuclease activity when the hydrogen bonding function of Asp^{549} is lost by truncating α-helix E′ to Val^{552} (Ghosh et al., 1995). These possibilities might be resolved by a mechanism requiring co-ordination of a second metal at site B (via Asp^{549} and Asp^{443}) and its participation in exonuclease function. With respect to our data with p66$^{E\rightarrow Q}$/p51 RT, Mn^{2+} may recruit the carboxylate

group *and* carbonyl oxygen of Asp^{443}. Recruiting the carbonyl oxygen of Asp^{443} would then have the consequence of eliminating metal binding at site B. In the case of heterodimer RT containing an 8-amino acid deletion of p66, loss of hydrogen bonding between Asp^{549} and Ser^{553} could also impair binding at site B, while allowing Mg^{2+} to coordinate Asp^{443}, Glu^{478}, and Asp^{498}. The consequence might again be retention of endoribonuclease activity (mediated by metal at site A) and loss of directional processing activity (mediated through metal at site B).

While the one- or two-metal ion-mediated hydrolysis of an RNA−DNA hybrid by HIV-1 RNase H still requires further study, the data presented here nonetheless provides evidence that RNase H activities can be modulated, the consequence of which is loss of DNA strand transfer function. The possibility of developing therapeutic agents which function by uncoupling endoribunuclease and directional processing activities should therefore be given consideration.

SUPPORTING INFORMATION AVAILABLE

Three figures showing coordination of Mn^{2+}-dependent endoribonuclease function with DNA synthesis activity of mutant $p66^{E\rightarrow Q}$/p51, DNA-dependent DNA synthesis, and RNA-dependent DNA synthesis (3 pages). Ordering information is given on any current masthead page.

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