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RNase H domain mutations affect the interaction between Moloney murine leukemia virus reverse transcriptase and its primer-template

(DNA polymerase/processivity/dimerization/retroviral replication)

ALICE TELESNITSKY AND STEPHEN P. GOFF

Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, NY 10032

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ABSTRACT **The active sites for the polymerase and nuclease activities of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) reside in separate domains of a single polypeptide. We have studied the effects of RNase H domain mutations on DNA polymerase activity. These mutant RTs displayed decreased processivity of DNA synthesis. We also compared complexes formed between primer-templates and mutant and wild-type reverse transcriptase (RT). Although M-MuLV RT is monomeric in solution, two molecules of RT bound DNA cooperatively, suggesting that M-MuLV RT binds primer-template as a dimer. Some mutant RTs with decreased processivity failed to form the putative dimer.**

Retroviral reverse transcriptase (RT) contains two enzymatically distinct activities: a polymerase activity that synthesizes DNA using either RNA or DNA templates, and a nuclease activity, termed RNase H, that degrades the RNA strand of RNA·DNA hybrids (1). Both DNA polymerase and RNase H activities are required during retroviral replication to complete synthesis of a double-stranded DNA copy of retroviral genomic RNA (2). RNase H degrades the genomic RNA when it is complexed to nascent minus-strand DNA and performs such specialized functions as formation of the primer for plus-strand DNA synthesis and removal of minus-strand primer tRNA (3).

RTs of various retroviruses have different subunit structures (1). Unlike the heterodimeric RTs of human immunodeficiency virus (HIV) or the avian retroviruses, RTs of murine retroviruses such as Moloney murine leukemia virus (M-MuLV) are monomeric in solution (4, 5). Mutational analyses of M-MuLV RT have demonstrated that its two catalytic sites are separable genetically (6). Several M-MuLV RNase H domain mutants maintain full DNA polymerase activity, and most polymerase domain mutants retain normal levels of RNase H (6–9). It remains unclear whether the two activities are functionally coupled during retroviral replication or even whether a single RT molecule carries out both activities (10, 11). *In vitro*, polymerase and nuclease activities of retroviral RTs can function independently.

There is some evidence that mutations in the RNase H domain can affect polymerase activity. For example, one M-MuLV RT mutant, termed ΔC, forms shorter minus-strand DNA products than wild type, suggesting that ΔC RT is defective in elongation of DNA synthesis (12). In this report, we compare the products of DNA polymerization by three forms of M-MuLV RT that differ in their RNase H domains. We demonstrate that structural alterations in the RNase H domain of M-MuLV RT can affect the processivity of DNA synthesis without decreasing basic DNA polymerase activity. In a gel-shift assay, wild-type RT, but not structurally altered RNase H mutants, displayed cooperative primer template binding. We propose that M-MuLV RT dimerizes when it binds DNA and that the mutations described here interfere with this dimeric structure.

MATERIALS AND METHODS

Materials. RT was purified from bacterial lysates as described (12). The RT mutants mentioned here have been or will be described elsewhere (ref. 12; S. Blain and S.P.G., unpublished data). The specific activities of the enzyme preparations were 44, 99, and 112 units of DNA polymerase activity per μg of protein for wild-type, ΔC, and ΔRH RT, respectively (12).

Oligonucleotides (Genosys, Houston) were quantified by measuring absorbance at 260 nm. The concentrations of other single-stranded DNAs were estimated by hybridizing to known quantities of oligonucleotides. Radiolabeled nucleotides were from Amersham. Oligo(dT)$_{12-18}$, poly(A) (average chain length, 1 kb), and unlabeled nucleotides were from Pharmacia. In all experiments, RT molar concentrations are for wild-type RT, and equivalent units of mutant enzyme preparations were used. Primer-template concentrations are for primer 3′ ends.

Heteropolymeric Templates. Heteropolymeric DNA templates were derived from an M13-based subclone containing M-MuLV RT sequences. A 450-nt *Bgl* II/*Hin*dIII fragment of the *pol* gene on plasmid pNCA (13), which contains an intact M-MuLV provirus, was subcloned into M13mp18. The template was formed by annealing primer 26 (5′-GTGATGAATTTTCTAGAGGAGGGTAG-3′; 150 nM) with a 3-fold excess of the M13 derivative DNA in 50 mM Tris·HCl, pH 8/10 mM $MgCl_2$/50 mM NaCl for 10 min at 70°C and then allowing to cool to 25°C for 30 min.

Heteropolymeric templates of a defined length were generated by asymmetric PCR (14) on the M13 subclone described above, using primer 26 and excess reverse sequencing primer (5′-AACAGCTATGCCATG-3′) in the amplification. This generated a 224-nt single-stranded DNA identical in sequence to the initial template region of the M13 derivative. To generate the primer-template, 20 nM primer 26 was annealed to ≈2-fold molar excess of the template strand as described above.

Gel-Shift Assay Templates. Gel-shift assay 31–46 template consisted of two cDNA oligonucleotides: primer 31 (5′-CCAGCAGTAAGTTGTTAACGTACTGTAGCAG-3′) and primer 46 (3′-GGTCGTCATTCAACAATTGCATGACATCGTCGTCCGTTCCTTCCAG-5′). These oligonucleotides were annealed by incubating each oligonucleotide (4 μM) as described above.

The longer gel-shift assay template was generated by annealing two separated single-stranded DNAs: the 322-bp *Pvu* II/*Pvu* II fragment of pUC19 and the longer *Pvu* II/*Xba* I fragment of pUC19. Strand separation was performed by standard methods (15), and approximately equal concentrations of the two complementary strands were annealed as

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Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; M-MuLV, Moloney murine leukemia virus; DTT, dithiothreitol.

described above. The resulting template was a 208-nt duplex with an additional 114 nt on the template strand.

Reverse Transcription Assays. Homopolymer assays for RT activity, which measure incorporation of TMP using oligo(dT)–poly(A) primer-templates, were performed under standard conditions (5). In heteropolymeric template assays, 8-μl reaction mixtures containing 60 mM Tris·HCl (pH 8), 75 mM NaCl, 7.5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 μM [α-^{32}P]dATP (400 Ci/mmol; 1 Ci = 37 GBq), plus 250 μM dGTP (only in Fig. 3*B*) and the indicated template concentrations were incubated at 37°C. After 7 min, 2.5-μl aliquots were removed to tubes containing prewarmed elongation mixture (60 mM Tris·HCl, pH 8/75 mM NaCl/7.5 mM $MgCl_2$/5 mM DTT/500 μM each dNTP) with or without oligo(dT)–poly(A), and incubation continued for 10 min. Products were recovered and applied to gels.

Gel-Shift Assays. Eight-microliter reaction mixtures contained 1.4 pmol of RT and 60 mM Tris·HCl (pH 8), 75 mM NaCl, 7.5 mM $MgCl_2$, 5 mM DTT, and the indicated 31–46 template concentrations. After 2 min at 37°C, 2 μl of prewarmed 60 mM Tris·HCl, pH 8/75 mM NaCl/7.5 mM $MgCl_2$/5 mM DTT/2.5 μM [α-^{32}P]dCTP (3000 Ci/mmol), and, where indicated, oligo(dT)–poly(A) at 50 μM oligo(dT) primer was added. After 4 min of further incubation, 1.5 μl of 60% sucrose was added and samples were applied to a 5% acrylamide/0.13% bisacrylamide gel cast and run in 25 mM Tris·HCl/162 mM glycine, pH 8.4, and run at 150 V at 4°C.

RESULTS

RNase H Mutant RTs. We used homology alignments and a predicted M-MuLV RNase H domain structure to design RNase H mutations (16–18). Mutant ΔRH is a truncated form of RT that lacks the entire RNase H domain (12). Mutant ΔC contains an 11-amino acid deletion in its RNase H domain (12) and lacks the third RNase H domain α-helix [termed the C helix (18)]. ΔC RT retains most of its RNase H activity (12). Mutant D524N (Asp-524 → Asn) contains a point mutation in a highly conserved aspartate residue proposed to function in RNase H catalysis (18, 19) and known to be important for viral infectivity (20). DNA polymerase activity, as measured by incorporation in the homopolymer assay, is not decreased for any of these RNase H mutants.

To compare the properties of various RTs, we purified the proteins by column chromatography from lysates of *Escherichia coli* expressing each variant. The preparations were >90% pure and free of *E. coli* RNase H (12). The enzyme encoded by the parental construct was identical in sequence to wild-type viral M-MuLV RT (12), and the mutant RTs differ from wild type only in their RNase H regions.

Homopolymer Assay Products. To determine the lengths of DNAs synthesized by various RTs on homopolymer templates, we performed reactions on an oligo(dT)–poly(A) primer-template (21) and examined the products by gel electrophoresis (Fig. 1). These reactions were performed with vast template excess, using the same amount of DNA polymerase activity in each reaction. As previously observed, wild-type RT makes longer-than-template length products in the homopolymer assay (22). The ΔRH polymerase formed only very short products, and ΔC RT generated intermediate length products. Product size distribution was unaffected when the enzyme concentration was decreased 10-fold (data not shown), showing that the difference was not due to small variation in the amount of input enzyme. The short ΔRH RT products were not due to a failure to displace primers on oligo(dT)–poly(A), which is essentially a gapped duplex, since decreasing the ratio of oligo(dT) to poly(A) 10-fold did not increase product length (data not shown). Other investigators recently compared the properties of commercially available wild-type and single domain M-MuLV RT on oligo(dT)–poly(A) and made similar observations (23).

Processivity on Heteropolymeric Templates. We compared these enzymes' DNA polymerase activities on heteropolymeric templates. Although RT synthesizes longer products on RNA than on DNA (24), we used DNA templates to avoid any effects of variations in RNA hydrolysis on DNA synthesis. Products formed by each RT during a single processive cycle were compared in a template challenge experiment (Fig. 2). RT was prebound to primer-template, and DNA synthesis was initiated by simultaneous addition of deoxyribonucleotides in the presence or absence of a 350-fold molar excess of challenge template. Although the processivity of all three forms of RT was very low under these conditions, wild-type RT formed the longest products, ΔC RT gave products of intermediate length, and ΔRH RT made the shortest products. Similar differences in processivity were

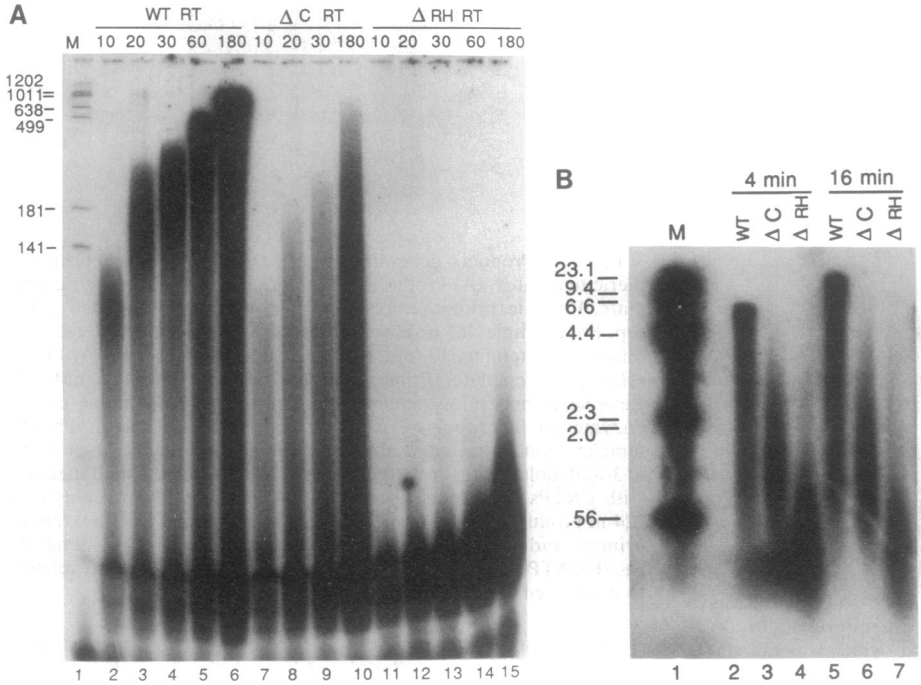

FIG. 1. Homopolymer assay time course. (*A*) Autoradiogram of polyacrylamide gel comparing products generated at short time points. Numbers above lanes indicate time of incubation in sec. Equal fractions of each reaction mixture were loaded in each sample lane. Lanes: 1, markers (sizes are in nt); 2–5, products generated by wild-type (WT) RT; 6–9, ΔC RT products; 10–15, ΔRH RT products. (*B*) Autoradiogram of alkaline agarose gel comparing products at longer time points. Lanes: 1, markers (sizes are in kb); 2 and 5, wild-type (WT) RT products; 3 and 6, ΔC products; 4 and 7, ΔRH products. For each time point, equal fractions of reaction products were loaded for each enzyme.

observed on other DNA templates (data not shown). Thus, RNase H mutations affect processivity. Since no RNA hydrolysis occurs in these reactions, the basis of the effects of these mutations is presumably structural rather than enzymatic. All three RTs formed longer products with excess enzyme (Fig. 3), but ΔC and ΔRH required a higher molar excess than wild type. With an enzyme/primer-template ratio of 500:1, all three RTs could synthesize a 224-nt product (Fig. 3*B*), and at various lower ratios correspondingly shorter products were formed (data not shown).

RT Plus Primer-Template Complexes. Gel-shift assays were used to determine whether these forms of RT differ in nucleic acid binding. RT was incubated with a short primer-template, composed of two oligonucleotides forming a duplex of 31 bp with an additional 15 nt on the template strand, termed the 31–46 primer-template. Complexes were radiolabeled by providing the first template-complementary nucleotide, [α-^{32}P]dCTP and analyzed by electrophoresis on nondenaturing polyacrylamide gels (Fig. 4). The pattern of retarded complexes was qualitatively the same whether or not oligo(dT)–poly(A) competitor was added simultaneously with the complex-labeling nucleotide (Fig. 4, compare lanes 1–4 with lanes 5–8). This suggests that the retarded bands resulted from complexes of RT bound productively at the primer 3′ terminus. The same pattern of retarded bands was also observed with a longer (54–64 nt) primer-template.

Both the wild-type and D524N RTs yielded two distinct retarded bands. We propose that the faster-migrating retarded band represents a complex containing a single molecule of RT and that the more slowly migrating retarded band reflects complexes with two molecules of RT bound to a single primer-template. Unlike wild-type RT, ΔRH and ΔC RTs each generated predominantly a single retarded band. The ΔRH band showed significantly higher mobility, consistent with the lower molecular weight of the mutant protein.

We examined the formation of these retarded complexes as a function of the RT/primer-template ratio. Wild-type RT forms the more slowly migrating complex even in the presence of a 25-fold molar excess of primer-template, and thus two molecules of RT appear to bind cooperatively (see Fig. 5*A*, lane 12). With enzyme excess, both ΔRH and ΔC formed secondary, more slowly migrating complexes that probably resulted from binding of a second RT molecule to a single primer-template (see Fig. 5*A*, lanes 1 and 5).

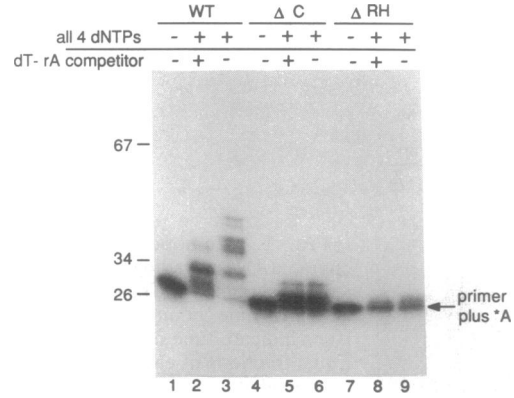

FIG. 2. Products of a single cycle of processive DNA synthesis on a heteropolymeric template. RT was prebound to the template at an enzyme/primer-template ratio of 3:1. Primer template concentration, 10 nM. Lanes: 1, 4, and 7, nonelongated primer; 2, 5, and 8, products of a single cycle of DNA synthesis, generated by simultaneous addition of dNTPs and of oligo(dT)–poly(A) competitor template at 350-fold excess relative to enzyme; 3, 6, and 9, products generated without competitor. Marker sizes are in nt. Primer plus *A indicates mobility of unextended primer strand, which was radiolabeled at its 3′ end with [α-^{32}P]dATP. WT, wild type.

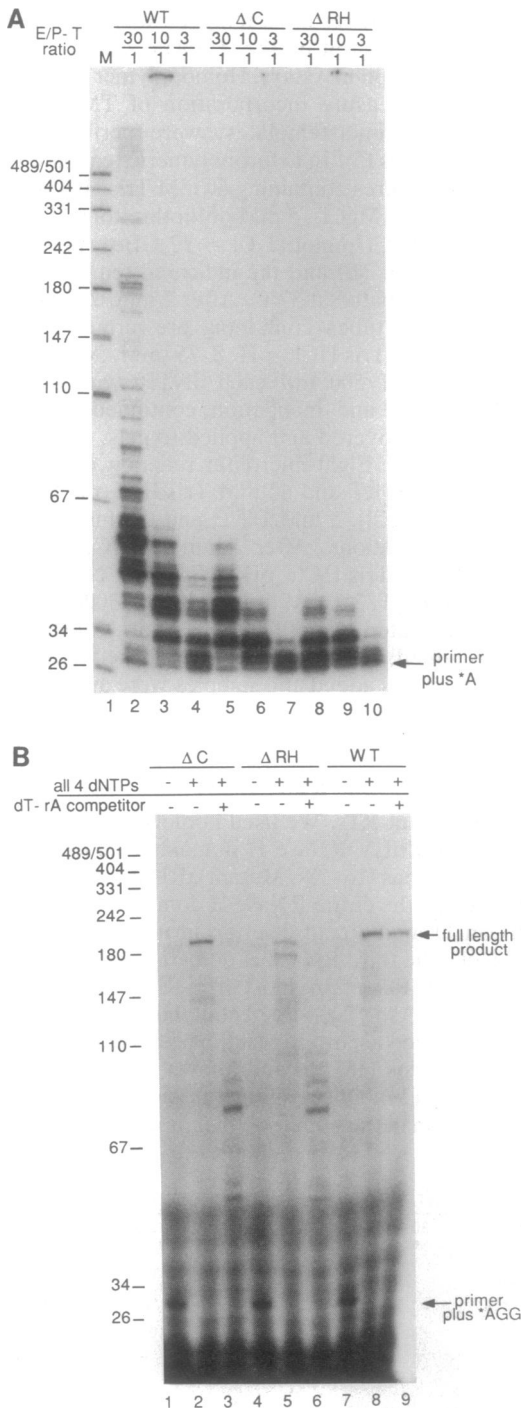

FIG. 3. Products generated by excess enzyme on heteropolymeric templates. (*A*) Products synthesized on heteropolymeric template. Enzyme/primer-template (E/P-T) ratios are indicated at the top. For example, RT products in lane 2 were generated in a reaction mixture containing 30-fold molar excess of wild-type (WT) RT relative to template. Primer-template concentration, 10 nM. (*B*) Reverse transcription products formed at 500-fold molar excess enzyme on heteropolymeric template of defined length. Primer-template concentration, 2 nM. Where indicated, oligo(dT)–poly(A), at 3-fold molar excess relative to enzyme, was added simultaneously with dNTPs. Full length product indicates mobility of completed 224-nt product. Primer plus *AGG indicates mobility of unextended primer, radiolabeled at a single position near its 3′ end by [α-^{32}P]dATP. Higher level of background radioactivity in *B* relative to *A* resulted from differences in sample processing.

The secondary complex that formed on the short primer-template with excess ΔC differed in mobility from the second-

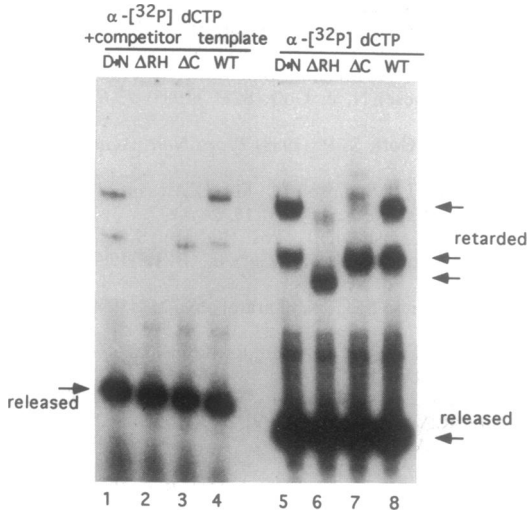

FIG. 4. Gel-shift assay of complexes on a short primer-template. Enzyme/primer-template ratio, 1:7. Comparisons of phenol-extracted and nonextracted reaction mixtures demonstrated that the lower band in each lane contains labeled 31–46 primer-template not bound to enzyme (data not shown); hence, this band is designated released product. Lanes: 1 and 5, complexes formed with D524N RT; 2 and 6, ΔRH RT; 3 and 7, ΔC RT; 4 and 8, wild-type (WT) RT.

ary band that resulted from cooperative wild-type RT binding (Fig. 5*B*, compare retarded bands in lanes 4 and 5). We postulate that the two wild-type RT molecules cooperatively bound to a single primer-template are associated in an enzyme dimer bound at the primer terminus. However, the differences in mobilities of wild-type and ΔC RT complexes suggest that these complexes differ structurally. We postulate that when two molecules of ΔC or ΔRH RT bind noncooperatively to a single template, as they do in high enzyme excess, these two molecules are not parts of an enzyme dimer.

DISCUSSION

We have examined several aspects of the DNA polymerase activity of M-MuLV RT RNase H domain mutants. Unlike HIV RNase H mutants, many of which have low DNA polymerase activity (25), the M-MuLV RT RNase H mutants examined here are fully active DNA polymerases when assayed by measuring TMP incorporation on oligo(dT)–poly(A) primer-templates (12). However, the products synthesized on oligo(dT)–poly(A) are significantly shorter than those of wild-type RT, and the mutants are less processive than wild-type RT on heteropolymeric templates.

Viruses harboring these mutant RTs are not infectious. However, it is not clear whether this is due to processivity defects or whether other aspects of retroviral replication such as RNase H specificity are also affected. Within retroviral particles, there is ≈50-fold more RT than viral RNA, and viral nucleocapsid protein or other aspects of the capsid environment may aid processivity (26, 27).

Low-processivity forms of RT may increase prematurely paused DNA products during replication. One model for retroviral recombination involves template switching during reverse transcription (28, 29), and since RT pausing is a likely prerequisite to template switching (30), it would be interesting to study the effects of RT processivity mutants on the rate of retroviral recombination.

Since simultaneous DNA synthesis and template hydrolysis have been observed *in vitro*, the active sites of the DNA polymerase and RNase H domains are probably both engaged on the template during DNA synthesis (10). This interaction of the RNase H domain with the template may stabilize the interaction between RT and its primer-template and aid processivity. This model is consistent with results of a comparison of primer binding affinities of wild-type HIV RT p66/p51 heterodimer versus that of the p51 homodimer, which lacks the RNase H domain (31). Both forms of HIV RT bind short primers with equal affinity, but the presence of the RNase H domain results in ≈1000-fold lower K_d for primers >15 nt. This coincides with estimates of the distance between polymerase and nuclease active sites for actively reverse-transcribing RT made by evaluating the length of RNA template strand impervious to RNase H digestion (10, 32, 33).

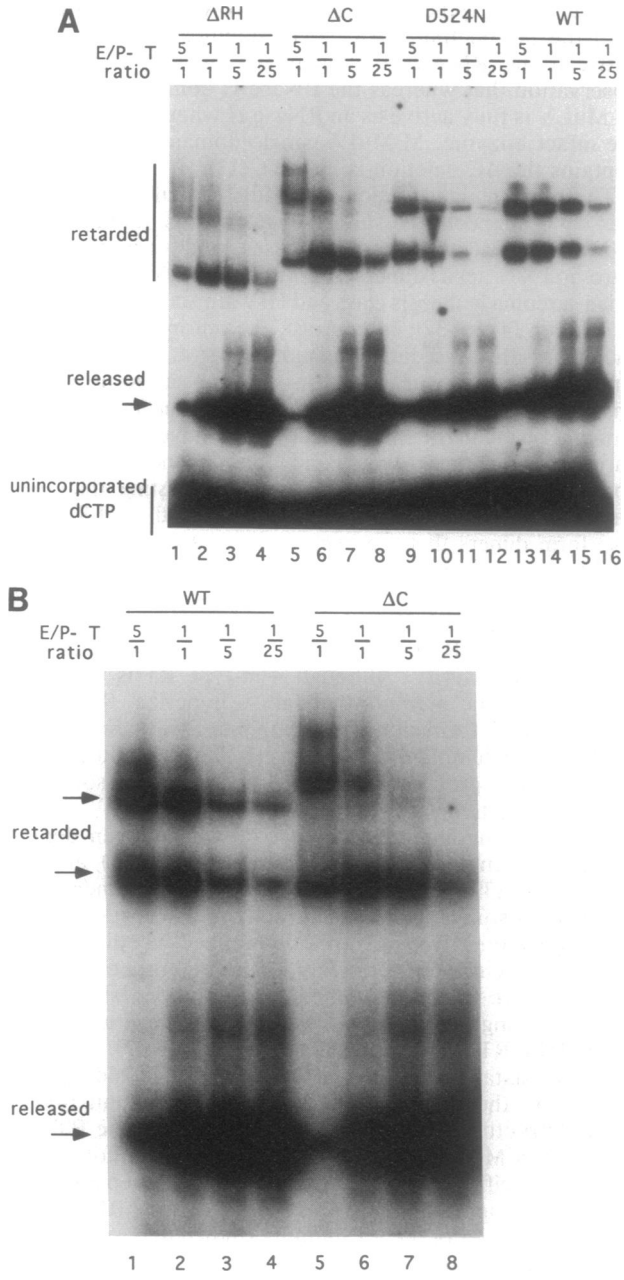

FIG. 5. RT plus primer-template complexes formed at various enzyme/primer-template (E/P-T) ratios. (*A*) Effects of varying E/P-T ratio. Ratios are indicated. For example, lanes 1, 5, 9, and 13 contain a 5-fold excess of enzyme relative to primer-template. (*B*) Comparison of gel mobilities of wild-type (WT) and ΔC RT complexes. Lanes: 1–4, wild-type RT; 5–8, ΔC RT.

The ΔC deletion may decrease processivity because the basic residues in the C helix ordinarily stabilize enzyme/primer-template interactions. The C helix region of both M-MuLV RT and *E. coli* RNases H is rich in basic amino acids and replacing them decreases substrate binding affinity

without affecting the rate of RNA hydrolysis (34). Consistent with the notion that the C helix stabilizes the interaction between the RNase H domain and nucleic acids is the observation that whereas the RNase H domain of wild-type M-MuLV is fully active as an RNase H when separated from the intact enzyme, M-MuLV single domain RNase H that contains the ΔC deletion is inactive (12).

The apparent dimerization of M-MuLV when bound to DNA was an unexpected result. However, this finding unites M-MuLV RT with other RT classes, and suggests that all retroviral RTs act as dimers. RTs from such retroviruses as the avian sarcoma leukeosis class and HIV are stable heterodimers in solution (4, 35–37). In contrast, the murine retroviral RTs such as M-MuLV sediment as monomers (4, 5). The putative dimer observed in our gel-shift assays appears to be a rather unstable and low-affinity dimer, based on the presence of a monomer band in the same reactions. We have observed some variation in monomer/dimer band ratios depending on reaction conditions (data not shown), further suggesting that these putative dimer complexes are unstable. ΔC and ΔRH RTs may also form dimers that are even more unstable than those of wild-type RT and thus not detectable in our gel system.

Studies of additional RT mutants will be needed to explore whether cooperative DNA binding is required for processive DNA synthesis. RT dimerization is apparently not sufficient for high processivity. Although wild-type RT is more processive than the mutants that fail to dimerize, the D524N mutant, which forms long homopolymer assay products and binds primer-template cooperatively like the wild-type enzyme, is less processive than wild-type RT on some heteropolymeric templates (data not shown).

DNA binding-induced oligomerization has been demonstrated for enzymes such as Rep helicase (38) and Mu transposase (39). Each Rep helicase protomer binds DNA, and it is postulated that the protomers act sequentially in DNA unwinding. We have not determined whether each of the RT molecules cooperatively bound to a primer-template can be active in DNA synthesis. Crosslinking studies and subunit mixing experiments show that only the larger subunit of the HIV RT heterodimer is active (36, 40–42). Emerging structural data show that the second subunit is folded differently from the larger, catalytically active subunit (43). A similar structural alteration may also occur in one RT molecule when M-MuLV RT dimerizes. It may be noteworthy that the specific activity of our wild-type RT preparations is consistently approximately half that of ΔC or ΔRH RT.

The properties of the RT mutants studied here suggest that the RNase H region is somehow involved in dimerization. Although the ΔC deletion destroys cooperative RT binding, it is not clear whether the ΔC region is directly involved in dimerization. Preliminary results with two additional RNase H structural mutants, each of which (like ΔC) is predicted to maintain its RNase H catalytic core, show that these enzymes bind primer-templates cooperatively. These properties of these additional mutants—one a deletion between predicted β-strand 5 and helix E of the RNase H domain and another an insertion between helix D and β-strand 5 [the secondary structure designations as in Yang *et al.* (18)]—demonstrate that not all structural alterations of the RNase H domain affect dimerization and support the notion that the C helix region may play a direct role in cooperative DNA binding.

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