Characterization of RNA Strand Displacement Synthesis by Moloney Murine Leukemia Virus Reverse Transcriptase

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Colleen D. Kelleher and James J. Champoux‡
From the Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington 98195-7242

The RNase H activity of reverse transcriptase (RT) is presumably required to cleave the RNA genome following minus strand synthesis to free the DNA for use as a template during plus strand synthesis. However, since RNA degradation by RNase H appears to generate RNA fragments too large to spontaneously dissociate from the minus strand, we have investigated the possibility that RNA displacement by RT during plus strand synthesis contributes to the removal of RNA fragments. By using an RNase H− mutant of Moloney murine leukemia virus (M-MuLV) RT, we demonstrate that the polymerase can displace long regions of RNA in hybrid duplex with DNA but that this activity is approximately 5-fold slower than DNA displacement and 20-fold slower than non-displacement synthesis. Furthermore, we find that although certain hybrid sequences seem nearly refractory to the initiation of RNA displacement, the same sequences may not significantly impede synthesis when preceded by a single-stranded gap. We find that the rate of RNA displacement synthesis by wild-type M-MuLV RT is significantly greater than that of the RNase H− RT but remains less than the rate of non-displacement synthesis. M-MuLV nucleocapsid protein increases the rates of RNA and DNA displacement synthesis approximately 2-fold, and this activity appears to require the zinc finger domain.

Retroviral replication requires the single-stranded RNA genome of the virus to be converted into double-stranded DNA through a complex series of reactions termed reverse transcription. This process appears to be catalyzed solely by the viral reverse transcriptase (RT) that possesses the following two distinct enzymatic activities: a polymerase activity that synthesizes DNA using either RNA or DNA templates, and an RNase H activity that cleaves RNA in hybrid duplex with DNA.

The current model of reverse transcription proposes that the RNase H activity of RT is critical for several steps including degradation of the 5′ end of the RNA genome following minus-strand DNA synthesis to facilitate the first jump, specific cleavage at the polypurine tract to create the plus strand primer, and removal of the plus and minus strand primers. Additionally, it is presumed that the RNase H activity is required to degrade the RNA genome following minus strand synthesis to free the minus strand DNA for use as a template during plus strand synthesis (reviewed in Ref. 3).

In vitro studies, however, suggest that RNA fragments that are too large to spontaneously dissociate from the minus strand remain following cleavage of the genome (4–11). Furthermore, evidence that plus strand synthesis in several retroviral systems is discontinuous demonstrates that stably annealed RNA fragments persist in vivo (3, 12–14). This raises the interesting possibility that reverse transcription requires a mechanism for the displacement of genomic RNA fragments during plus strand synthesis.

Most replicative polymerases require accessory proteins such as helicases and single-strand binding proteins (SSBs) to unpair the duplex region in front of the primer terminus during DNA synthesis (15). In contrast, studies from our laboratory and others (16–20) have demonstrated that RTs from several retroviral systems possess the capacity to catalyze displacement of the non-template DNA strand in the absence of accessory proteins, although the rate of synthesis appears to be roughly 3–12-fold slower than that found during non-displacement synthesis on a single-stranded template. Similarly, both human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus (M-MuLV) RTs appear to possess at least a limited capacity to displace non-template RNA during synthesis on RNA-DNA hybrid templates (21), but this process has not been characterized in detail.

Since reverse transcription to yield full-length viral DNA in vitro has only been achieved in permeabilized virions or ribonucleoprotein complexes (22–24), it seems possible that one or more virion-associated accessory proteins are required for the complete reaction. A leading candidate for the role of an accessory factor is the viral nucleocapsid (NC) protein. NC is a small, basic protein that possesses either one or two zinc-finger motifs in conventional retroviruses. NC binds nucleic acids with some apparent cooperativity, shows a higher binding affinity for RNA over DNA with a preference for single strands, and promotes renaturation between complementary nucleic acid chains (25–31). These properties are reminiscent of those associated with SSBs (32–34), thus leading to the proposal that NC may serve to facilitate reverse transcription. Many studies have indicated that NC promotes the first and second template switches and is important during the initiation of reverse transcription from the tRNA primer (35–40), while other reports have suggested that NC improves the efficiency of synthesis during reverse transcription (39, 41–45).

In this study we have tested the ability of M-MuLV RT to catalyze RNA displacement synthesis in the absence or pres-
ence of the RNase H activity, and we have investigated the effects of NC on both DNA and RNA displacement synthesis. Our results indicate that RT has the capacity to displace RNA, but the rate is slower than that of DNA displacement synthesis and much slower than non-displacement synthesis. We find that M-MuLV NC facilitates displacement and that this activity is dependent on the zinc finger motif of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

SuperScript II (200μl) was purchased from Life Technologies, Inc. T4 DNA polymerase (3 units/μl), T4 polynucleotide kinase, Vent DNA polymerase, and restriction endonucleases were from New England Biolabs. Recombinant M-MuLV RT (200 units/μl) and Sequenase (13 units/μl) were from Amersham Pharmacia Biotech. Denaturing polyacrylamide gels (8.3 M urea) were prepared from Sequagel reagents (National Diagnostics).

**Nucleic Acids**

**Plasmids**—The recombinant phagemid pBSMLTR3− was generated as described previously for pBSMOLTR− (+ except for the long terminal repeat (LTR) insert was cloned into plasmid KSII (Stratagene) such that minus sense single-stranded DNA would be infected by infection with M13KO7 helper phage. The recombinant phagemid pGEMLTR2 contains a 645-bp fragment of the M-MuLV LTR isolated from pBSMLTR− (genomic position 7851 to 231) and cloned into pGEM3Zf+ (Promega Corp.). Single-stranded pGEMLTR2 DNA produced by helper phage rescue contained minus strand viral sequences. Phagemid pGEMLTR3 was generated by cloning a 744-bp EcoRI-BamHI LTR insert from pBSMLTR− into pGEM3Zf+ such that phage-released single-stranded DNA contained plus strand viral sequences. For brevity, nucleic acids derived from pGEMLTR2 and pGEMLTR3 will be referred as to LTR2 and LTR3, respectively. M13LTR2 was constructed by polymerase chain reaction amplification of the LTR insert (ssLTR2) from M13LTR2, the DNA was heated to 90 °C, slow-cooled in EcoRI buffer to anneal the complementary regions flanking the insert, and digested with 500 units of EcoRI, BamHI-linearized LTR2 DNA was subjected to electrophoresis on a 6% denaturing polyacrylamide gel to recover the single-stranded DNA oligonucleotides except R region of the M-MuLV genome (48), were kindly provided by J. L.

**Single-stranded DNAs**—Various single-stranded recombinant phagemid and phage DNAs were isolated by established procedures (46). Where indicated, the single-stranded phagemid DNA was linearized by restriction enzyme digestion after annealing an oligonucleotide that generated the restriction enzyme recognition site, followed by phenol/ chloroform extraction of the single-stranded insert (ssLTR2) from M13LTR2, the DNA was heated to 90 °C, slow-cooled in EcoRI buffer to anneal the complementary regions flanking the insert, and digested with 500 units of EcoRI in a final volume of 0.5 ml for 1 h at 37 °C. Following phenol/chloroform extraction, the 687-nt single-stranded EcoRI product was gel-isolated with QIAEX II (QIA- GEN) on a 0.7% agarose gel following the manufacturer’s protocol. Several single-stranded DNA fragment preparations were combined and further purified over an anion column (QIAGEN-tip 100) as specified by the manufacturer.

**Preparation of RNA**—For LTR2 RNA, BamHI-linearized LTR2 DNA was transcribed by T7 RNA polymerase as specified in the RiboMAX kit (Promega) except that prior to DNase I treatment, the reaction was treated with Escherichia coli alkaline phosphatase (−0.4 units/μg plasmid DNA) for 20 min at 37 °C with 5’-triphosphates (46). Full-length RNA transcripts were purified by electrophoresis on a 6% denaturing polyacrylamide gel and elution into 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) for 14 h at 25 °C. The eluate was filtered through a 0.2-μm syringe filter (Corning Glass Works), ethanol-precipitated in the presence of 0.3 M sodium acetate, and resuspended in TE. LTR3 RNA was generated using SP6 RNA polymerase (New England Biolabs, Inc.) following established procedures (46). Following phenol/chloroform extraction and ethanol precipitation, the RNA was treated for 20 min at 37 °C with E. coli alkaline phosphatase (2 units/μg RNA). SDS was added to 0.05%, and the RNA was extracted twice each with phenol and chloroform, ethanol-precipitated in the presence of 0.3 M sodium acetate, and resuspended in TE. To establish the efficiency of full-length RNA produced, 150 bp of transcribed RNA were carried out containing 36 μM UTP and 50 μCi of [α-32P]UTP. Although a detectable fraction of the LTR3 RNA appeared to be truncated (perhaps due to premature termination by SP6 RNA polymerase), >80% of the transcripts were >500 nt or larger. Therefore, all calculations involving RNA displacement on pGEMLTR3 used 500 bases as the effective end of the displacement region. All RNAs were stored at −80 °C.

**Oligonucleotides and 5’ End Labeling**

Synthesis and purification of RNA oligo IV have been described previously (47). DNA oligonucleotides R− and R+, corresponding to the R region of the M-MuLV genome (48), were kindly provided by J. L. Darlix (LaboRetro, INSERM, Lyon, France). DNA oligo IV was synthesized on a Biosearch 8600 DNA synthesizer, and the remaining DNA oligonucleotides were purchased from Macromolecular Resources. All DNA oligonucleotides except exo- R−, R+, BamHI, EcoRI-, and TermM13 were further purified by urea-polyacrylamide gel electrophoresis. DNA oligo II (5’-AGAAAAAGGGGGGAA-3’) and DNA and RNA oligos IV (5’-GAAAGCCCCAC-3’) correspond to sequence positions 7801–7816 and 7817–7829 of the M-MuLV genome, respectively (49). T7 primer (5’-GTGTAATTGTAACTAGCTACTATA-3’), T730 primer (5’-GGCGATTGAACTTGGCTC-3’), and SP6 primer (5′-CTGAAGTTTTACGACTATA-3′) annealed immediately upstream of the RNA transcription start site. DD oligos, LTR2Ne (5’-GGGCCGCTTAAGTA-3’) and LTR3hind (5’-GAATACTAAGCTTTG-3’) anneal immediately downstream of the T7 and SP6 primers, respectively. Gap-primer (DNA displacement), or TE (non-displacement) anneals to single-stranded LTR2 40 nucleotides upstream of the RNA transcription start site (see Fig. 4A). Bsm8I (5′-TCTGTGGGGATCCTCTAGA-3’) and Eco3’ (5′-ACTCTAGTTGCGCTCCACAC3’) create BamHI or EcoRI restriction sites when annealed to single-stranded LTR2 or LTR3, respectively. T7M13 (5′-CGCAAGTTGAGGCGTATTGAGG-3’) and TermM13 (5′-CGGATTCACCCACATAGTCGAG-3’) were used to polymerize template DNA to insert to pGEMLTR2 with the introduction of flanking EcoRI sites. When necessary, oligonucleotides were 5’-32P-end-labeled as described previously.2 The 100-μl DNA ladder (Life Technologies, Inc.) was 5’-32P-end-labeled by the polynucleotide kinase exchange reaction (50).

**Preparation of Primer-Templates**

**Short Oligonucleotide Primer-Templates**—To ensure that only the downstream oligonucleotides varied between the primer-template reactions, the oligonucleotides were annealed to the single-stranded template DNA in two stages. In the first stage, the end-labeled primer (oligo II) was annealed to the single-stranded DNA template (pBSMOLTR) at a molar ratio of 1:1.5 in 14× RT buffer (see below), by heating to 63 °C, and then slow-cooling to 14 °C. In the second stage, the annealed sample was divided into three parts to which were added either DNA oligo IV (DNA displacement), or TE (non-displacement) resulting in a ratio of primer-template:downstream oligonucleotide of 1:1.5:7.5 (see also Fig. 1A). The second stage annealing was performed under the same conditions as the first.

**Extended Primer-Templates**—The DNA displacement templates were prepared as follows (see also Fig. 2A): the DD oligo (corresponding to the 5’ end of the downstream non-template strand) was annealed to linear single-stranded DNA (or ssLTR2i for NC assays) at a 1:2 molar ratio (single-stranded DNA:oligonucleotide) in 2.3× Sequenase buffer (Amersham Pharmacia Biotech) by heating for 3 min at 65 °C and then incubating at 42 °C for 45 min. Reaction conditions were adjusted to 40 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 50 mM NaCl, 6 mM diithiothreitol (DTT), 0.2 mM dNTPs, and the DD oligo was extended with Sequenase at 37 °C for 30 min. The reaction was terminated by the addition of EDTA to 20 mM, extracted twice with phenol/chloroform and chloroform, and then ethanol-precipitated in the presence of 0.3 M sodium acetate. As a control for the efficiency of the DD oligo extension, a parallel reaction was carried out except the DD oligo:linear DNA ratio was −1:12, and the DD oligo was 32P-end-labeled. For the DNA displacement primer, the 5’-32P-labeled oligonucleotide primer was annealed to the DNA displacement template primer at a molar ratio of 1:2 (primer:template) in annealing buffer (200 mM KCl, 10 mM Tris-HCl, pH 7.5), by heating for 45 min at 67 °C and then for 30 min at 41 °C. The RNA displacement and non-displacement templates were prepared by combining the end-labeled primer with single-stranded linear DNA (or ssLTR2i for NC assays) and in vitro transcribed RNA (RNA displacement template, or TE (non-displacement template) at a molar ratio of 1:1.6:2.7 (primer:template:RNA) and annealing as described above (see also Fig. 2A).

**Displacement Synthesis Assays**

**Displacement Assays Using Oligonucleotide Primer-Templates**—Synthesis assays contained 10 μM primer in a 30-μl reaction volume. The annealed short oligonucleotide primer-templates (described above) were preincubated with 200 units of SuperScript II (SSII) in 1× reac-
Displacement Assays Using LTR2 and LTR3 Primer-Templates—
Synthesis assays contained 10 nM primer, 50 mM Tris, pH 8.3, 50 mM KCl, 6 mM MgCl₂, 5 mM DTT, 0.1 µg/ml bovine serum albumin, and 200 µM dNTPs (final volume 27–40 µl). For each reaction, the annealed primer-template combination was warmed to 37 °C in the presence of MgCl₂, DTT, and bovine serum albumin and, after the addition of 300–400 units of SSII, preincubated for 30 at 37 °C. Synthesis was initiated by the addition of dNTPs to a final concentration of 200 µM. At the indicated times 5-µl aliquots were added to 15 µl of 98% formamide, 6 mM EDTA, 0.04% xylene cyanole and analyzed by denaturing polyacrylamide gel electrophoresis. To compare directly M-MuLV RT versus SSII, or for control reactions using T4 DNA polymerase, the dNTPs were added prior to preincubation at 37 °C for 2 min, and synthesis was initiated by the addition of the enzyme. Median and maximum extension lengths were determined essentially as described elsewhere, except the unextendable primer (defined as the lowest amount of primer length radioactivity at any time point in the series) was subtracted from the total unextended primer in each sample. The median extension length was defined as the length at which half of the products were longer and half were shorter; the maximum length was arbitrarily taken as the length that exceeded 99% of the total products. Extension rates were based on multiple independent experiments and determined by least squares analysis of extension lengths plotted as a function of time.

RT Assays Involving NC—NC assays were carried out using the ssLTR2i template (described above) to avoid adding excess DNA to which NC would potentially bind non-productively outside of the template region. The 5'-32P-labeled primers used were as follows: T720 (1 nm), which anneals immediately upstream of the non-template RNA to create a nicked primer-template, and T7M13 (2.7 nm), which anneals 17 nt upstream of the non-template RNA to form a gapped template. The experiments were carried out as described above except that 1–10 pmol of NCP10 or NCdd were added per fmol of primer to the preincubation mixture. For the "no NC control," an equivalent volume of NC resuspension buffer (below) was added. When necessary, SSII was diluted in RT dilution buffer (20 mM Tris- HCl, pH 8.0, 1 mg/ml bovine serum albumin, 2 mM DTT, and 20% glycerol). Extension lengths were plotted as a function of time, and the rates were calculated from the steepest portion of each curve.

Comparison of Displacement and Non-displacement Synthesis on Extended Primer-Templates—To characterize further RNA displacement synthesis by SSII and to compare the rate of RNA displacement to DNA and non-displacement synthesis, extended primer-templates derived from the M-MuLV LTR were generated (Fig. 2A). Kinetic analysis of synthesis by SSII on the LTR2 primer-templates supported our previous observation that RNA displacement synthesis by RT was slower than DNA and non-displacement synthesis. Most primers were extended to the end of the linear template (655 nt) within the first 2 min during non-displacement synthesis (Fig. 2B, lane 5), while the displacement products accumulated less rapidly on the DNA and RNA displacement templates. Significantly, 655 nt product was not observed until the 5-min time point during DNA displacement (Fig. 2B, lane 11) or the 20-min time point during RNA displacement synthesis (Fig. 2B, lane 17). The calculated maximum rates of non-displacement, DNA displacement, and RNA displacement synthesis were 12.9, 2.4, and 0.63 nt/s, respectively. Surprisingly, we observed that during RNA displacement synthesis, the majority of the extension products were terminated by the addition of dNTPs to a final concentration of 200 µM MgCl₂, DTT, and bovine serum albumin and, after the addition of...
RNA Displacement Synthesis by Reverse Transcriptase

Fig. 1. Comparison of strand displacement with non-displacement synthesis by SSII on oligonucleotide primer-templates. A, RNA or DNA forms of the 13-base oligo IV were annealed immediately downstream of 5'-32P-labeled DNA oligo II to create a nicked primer-template for displacement assays, or oligo IV was omitted to generate a non-displacement template. Programmed synthesis allowed primer extension to proceed until the first template directed dTTP was required, 13 bases beyond the point of initiation. B, non-displacement (lanes 2–6), RNA displacement (lanes 7–11), or DNA displacement (lanes 12–16) primer-templates were used in time course assays in which programmed synthesis was catalyzed by SSII. Aliquots of the reaction were terminated at the time points indicated above each lane, and the products were separated on a 20% denaturing polyacrylamide gel. The unextended primer is shown in lane 1. Arrow indicates the position of the directed end point for programmed synthesis. C, the accumulation of full-length product (defined in this experiment as extensions to the first template) were separated on a 20% denaturing polyacrylamide gel. The unextended primer is shown in lane 1. Arrow indicates the position of the directed end point for programmed synthesis. C, the accumulation of full-length product (defined in this experiment as extensions to or beyond the programmed end point) on the non-displacement (open circles), DNA displacement (triangles), or RNA displacement (squares) templates is shown as a function of time.

remained stalled after only 1 to 4 bases had been added (Fig. 2B, lanes 13–17) compared with the rapid extension of primers through this region on the non-displacement and DNA displacement templates (Fig. 2B, lanes 3–7 and 8–12). Therefore, the resulting distribution of RNA displacement synthesis products was bimodal; at the 20-min time point, for example (Fig. 2B, lane 17), 50% of the products were stalled after 1 to 3 bases had been added while the other 50% of the terminations were distributed over the remaining 652 bases of the template. Due to this early stalling, the median RNA displacement synthesis rate was calculated to be roughly 150 times less than that of DNA displacement synthesis. A summary of the average median and maximum extension rates calculated from multiple independent experiments is shown in Table I.

To test whether the stalled intermediates remained extendable or were dead-end synthesis products, we added either additional SSII or the displacing polymerase, Sequenase, to the RNA displacement reactions after the initial 20 min incubation. Time points up to 2 h after the second addition of enzyme showed that the stalled products were extendable by Sequenase, but little if any additional extension by SSII was observed (data not shown).

As described above for the oligonucleotide primer-template reactions, T4 DNA polymerase was used as a control to confirm the structure of the templates. As expected, on the non-displacement template T4 DNA polymerase efficiently extended the primers to full-length product (Fig. 2B, lane 18) but failed to extend significantly the primers on the DNA and RNA displacement templates (Fig. 2B, lanes 19 and 20).

Efficiency of RNA Displacement Synthesis Initiation Is Sequence-dependent—To test whether the bimodal distribution of products observed with the LTR2 primer-template was characteristic of RNA displacement synthesis in general, a second set of extended primer-templates (LTR3) was generated. These templates were similar to those shown in Fig. 2A, except that the sequence of the primer and the template downstream from the nick differed from the LTR2 templates. Fig. 3 shows a time course of synthesis by SSII over the first ~75 nt on the LTR3 templates. As with the LTR2 templates, the primers were efficiently extended during non-displacement and DNA displacement synthesis (Fig. 3, lanes 2–4 and lanes 5–10); by the 15-s time point for non-displacement (Fig. 3, lane 2) or the 5-min time point for DNA displacement synthesis (Fig. 3, lane 8), no significant stalled products remained within the first 10 bases downstream from the primer. The accumulation of stalled products in the same region at the 5-min time point was greater during RNA displacement (Fig. 3, lane 14), but they did not persist to later time points (lanes 15 and 16). The reduced stalling yielded a median rate for RNA displacement on the LTR3 template that was 5-fold greater (0.01 nt/s) than that with the LTR2 template. The maximum rates of non-displacement, DNA displacement, and RNA displacement synthesis on the LTR3 templates were 15.1, 3.2, and 0.88 nt/s, respectively, and thus similar to the maximum rates determined for the LTR2 templates.

Initiation of RNA Displacement on a Gapped Template—To determine whether the stalling observed on the LTR2 RNA

| Template | Maximum rate Mean nt/s | Maximum rate Mean (+/-) nt/s |
|----------|-------------------------|-----------------------------|
| Nondisplacement<sup>a</sup> | 11.60 | 2.87 |
| DNA displacement<sup>a</sup> | 2.98 | 0.43 |
| RNA displacement<sup>a</sup> | 0.60 | 0.12 |

<sup>a</sup> Based on five independent experiments.

<sup>b</sup> Based on three independent experiments.

<sup>c</sup> Based on two independent experiments.
FIG. 2. Template design and displacement synthesis assays on extended LTR2 primer-templates. 

A, shown schematically are the different primer-templates used in the LTR2 synthesis assays. DNA strands are depicted by straight lines and RNA strands by wavy lines. The template strand in each assay was linearized single-stranded LTR2 DNA. The template sequence downstream of the primer corresponds to 5 bases of vector followed by minus sense M-MuLV LTR DNA. 

B, synthesis by SSII on non-displacement (lanes 3–7), DNA displacement (lanes 8–12), and RNA displacement (lanes 13–17) templates was terminated at the time points (in minutes) indicated above each lane. As a control, synthesis by the non-displacing T4 DNA polymerase was measured at 20-min time points on identical non-displacement (lane 18), DNA displacement (lane 19), or RNA displacement (lane 20) templates. Products were separated on a 6% denaturing polyacrylamide gel. Lane 2 and arrows show the position of unextended primer. Size markers are shown in lane 1 and indicated at the left in nucleotides.
displacement template was affected by the position of the primer relative to the RNA non-template strand, a gapped RNA displacement template was created (Fig. 4A). Synthesis from the priming oligonucleotide (T7primer) used in the LTR2 studies (above) was compared with synthesis from an alternate primer (gap-primer) that annealed 40 bases upstream of the non-template RNA. This configuration does not change the sequence at which RNA displacement is initiated and thus allowed us to compare directly the synthesis initiating at a nick with that initiating at a gap.

When RNA displacement was preceded by non-displacement synthesis (gap configuration), stalling during the initiation of RNA displacement was reduced significantly. The arrows in Fig. 4B indicate the positions of the first base of RNA displacement (+1 position) on the gapped template (Fig. 4B, lanes 4–8) and on the nicked template (Fig. 4B, lanes 13–17); products migrating at or above the arrows reflect synthesis requiring RNA displacement. To minimize the contribution of products resulting from non-displacement synthesis through the gapped portion of the template, the 5- and 20-min time points were used to analyze stalling at the +1 position with the two templates. At the 5-min time point, 9.9% of the RNA displacement product was stalled at +1 on the gapped template, whereas 42.8% was stalled in the analogous position on the nicked template; the corresponding values for the 20-min time point were 3.7 and 16.8%, respectively.

Unexpectedly, the pausing pattern in the single-stranded region of the gapped template differed dramatically from the pausing over the same sequence with the non-displacement template. Significant pauses were observed at positions −7 to −1 (relative to the start of RNA displacement) on the gapped template (Fig. 4B, lanes 4–7, vertical line on left) that were absent on the non-displacement template (Fig. 4B, lanes 1 and 2). Similar differences were observed when pauses in the same region on a gapped DNA displacement template were compared with non-displacement products (data not shown). The basis for these pauses on what should be identical stretches of single-stranded DNA is not clear (see “Discussion”).

RNA Displacement Synthesis by Wild-type RT—Since the RNase H activity of RT is predicted to cleave the genomic RNA prior to plus strand synthesis in vivo, it was of interest to investigate the extent to which limited RNase H activity might affect the rate of RNA displacement synthesis by RT in our in vitro assay. DNA synthesis catalyzed by M-MuLV RT on the LTR2 primer-templates was compared with that catalyzed by SSII under identical conditions. On the RNA displacement template (Fig. 5A), accumulation of full-length products was observed as early as the 2-min time point when synthesis was catalyzed by M-MuLV RT (Fig. 5A, lane 10) but not until the 20-min time point with SSII (Fig. 5A, lane 7). For both SSII and M-MuLV RT, significant pausing was observed during the first 2 bases of addition. At the earliest time point, the amount of product stalled from the primer up to the +2 position was nearly identical for the two enzymes (Fig. 5A, lanes 3 and 8), but at later time points, these pauses were more effectively resolved in reactions containing RNase H activity (lanes 10–12) compared with those lacking it (lanes 5–7). A plot of the maximum extension rate by M-MuLV RT on the RNA displacement template shows an increasing slope up to the 5-min time point, after which the end of the linear template was approached (Fig. 5B). The initial extension rate by M-MuLV RT of 0.8 nt/s increased to a maximum of 2.5 nt/s between the 2- and 5-min time points. Synthesis by SSII on an identical template and under the same conditions yielded a linear extension rate of 0.6 nt/s. As expected, the rates of synthesis by SSII and M-MuLV RT on the DNA displacement or non-displacement templates were very similar (Fig. 5B).

Effect of M-MuLV NC on RNA Displacement Synthesis—We investigated the effect of M-MuLV NC on displacement synthesis by RT using chemically synthesized NC and mutant NC proteins. To test the functional activity of our NC preparations, we performed a standard annealing assay (48) in which the capacity of the protein to promote hybridization between complementary DNA strands was monitored. The 56-residue M-MuLV NC protein, NCP10, contains a single zinc coordination site (zinc finger) flanked by basic regions important in nucleic acid annealing (51). The NCdd mutant contains a Gly-Gly linker in place of the deleted zinc finger, and the NC-(19–53)
Fig. 4. Comparison of RNA displacement synthesis on gapped versus nicked primer-templates. A, shown schematically are the primer-templates used to compare synthesis on the gapped and nicked templates using linearized single-stranded LTR2 DNA as the template strand. The nicked template is identical to the LTR2 RNA displacement template (see Fig. 2A). The gap-primer anneals to the single-stranded DNA template 40 bases upstream from the 5' end of the RNA, leaving a 40-base single-stranded “gap” between the end-labeled primer and the non-template RNA strand. B, synthesis on non-displacement (lanes 1–3), gapped (lanes 4–8), and nicked (lanes 13–17) templates by SSII was terminated at the time points (in minutes) indicated above each lane, and the products were analyzed on a 6% denaturing polyacrylamide gel. The positions of the first base of RNA displacement are indicated by the arrows and, for the gapped template, were determined by reading the adjacent sequencing ladder (lanes 9–12) generated using 5'-32P-labeled gapped-primer. The vertical line (at left) marks the position of a series of pauses on single-stranded DNA unique to the gapped template.

DISCUSSION

In the present study, we have analyzed the capacity of MuLV RT to displace RNA during synthesis on RNA-DNA hybrid duplexes. In the absence of RNase H activity, RT carried out RNA displacement synthesis on either short oligonucleotide or extended hybrid primer-templates, but the rate of synthesis was lower than during either DNA displacement or non-displacement synthesis. These findings are consistent with

Mutant lacks the zinc finger as well as residues from the N and C termini. NCP10 and NCdd have been reported to promote nucleic acid annealing in vitro, whereas NC-(19–53) lacks annealing activity (52). In the control reaction without added protein a low level of background annealing was observed after incubation of the complementary 68-mer oligonucleotides at 37 °C for 5 min (Fig. 6, lane 2); heating and slow cooling of the oligonucleotides promoted nearly 100% duplex formation (Fig. 6, lane 3). Annealing was promoted by NCP10 in a dose-dependent manner (Fig. 6, lanes 7–9) with ~100% of the product migrating as duplex at the highest NCP10 concentration tested (Fig. 6, lane 9). Likewise, NCDd promoted annealing to a similar extent at equivalent molar concentrations (Fig. 6, lanes 10–12). Consistent with previously reported results, NC-(19–53) appeared to have little or no effect on the rate of annealing of the oligonucleotides (Fig. 6, lanes 4–6).

NCP10 and the NC mutants were tested for their effect on RNA displacement synthesis by RNase H RT. Titration assays were carried out by adding increasing concentrations of NC to the LTR2 RNA displacement assay prior to the addition of SSHI and dNTPs. Synthesis products from reactions containing either NCDd (Fig. 7, lanes 8–11) or NC-(19–35) (data not shown) appeared identical to mock reactions in which no NC protein was added (Fig. 7, lane 9). The extension products from reactions in which low concentrations of NCP10 were added appeared the same as the no NC control (compare Fig. 7, lanes 4 and 5 to lane 3), whereas the proportion of longer products increased in reactions with higher NCp10 concentrations (Fig. 7, lanes 6 and 7). The NC:nT ratio required to effect this shift was approximately equivalent to that found to promote duplex formation in the NC annealing reactions (Fig. 6 and data not shown). Of particular note, it appeared that the stalling consistently observed within the first 4 bases of RNA displacement synthesis on LTR2 was reduced in the presence of NCP10, and the amount of radioactivity migrating at ~100–150 nt increased (compare Fig. 7, lanes 6 and 7 with lane 3).

To characterize the extent to which NC facilitates RNA displacement synthesis by SSHI and to test the effect of NC on DNA displacement and non-displacement synthesis, time course assays were carried out in the absence of NC or in the presence of equivalent concentrations of NCP10 or NCdd. The presence of NCP10 or NCdd had no effect on the rate of extension during non-displacement synthesis (Fig. 8A), and analysis of the gel from which the rates were calculated revealed no qualitative difference when NC was added (data not shown). As was found for RNA displacement, NCP10 facilitated DNA displacement synthesis by SSHI, whereas NCDd did not (Fig. 8B); the maximum rate of DNA displacement synthesis in the presence of NCP10 was 1.7-fold greater than in the absence of NC and 1.9-fold greater than when NCdd was added. During RNA displacement synthesis (Fig. 8C), NCP10 improved the maximum extension rate of SSHI by 1.7- and 1.8-fold over that observed in the absence of NC or with NCdd, respectively. NCP10 increased the median rate of RNA displacement synthesis by 2-fold, while no significant change in the median rate of DNA or non-displacement synthesis was observed (data not shown).
those of Fuentes et al. (21) who showed that both HIV-1 and M-MuLV RTs lacking RNase H activity could displace a short RNA oligonucleotide during DNA synthesis.

We estimated the maximum rate of synthesis by SSII (RNase H$^2$RT) to be 0.6 nt/s during RNA displacement, a rate 5-fold slower than that of DNA displacement synthesis and 19-fold slower than non-displacement synthesis (Table I). However, the median rate of RNA displacement synthesis was approximately 750 times slower than non-displacement synthesis due to the substantial stalling that occurred during the first 4 bases of synthesis beyond the initial primer. Qualitatively it appears that synthesis was strongly inhibited during the first several bases of extension but that once beyond this point, the nascent chains were readily elongated.

We were interested in determining whether the stalling observed during initiation on the LTR2 template was a general property of RNA displacement synthesis by RT. If so, it could indicate that RT has a very limited capacity to displace RNA. Alternatively, we considered the possibility that factors such as sequence context or the initiation of displacement synthesis at a nick may have contributed to the stalling. The former possibility was addressed with the LTR3 primer-template pair. Alteration of both the primer and the downstream hybrid sequence led to a significant decrease in the amount of product stalled at initiation, results which concurred with the pattern of pausing observed with the oligonucleotide primer-templates. Thus initiation on the LTR2 template appears to be unusually inefficient. The similarity between the maximum rates of synthesis on the two extended primer-templates, however, provided strong evidence that displacement of RNA by RT is significantly slower than displacement of DNA under otherwise identical conditions. This conclusion is supported by the relative rates estimated from assays using the oligonucleotide primer-templates.

Why is RNA displacement more difficult than DNA displacement? If displacement synthesis is merely a passive process displacement template (see Fig. 2A) was terminated at the time points (in minutes) indicated above each lane. The products were resolved on a 6% denaturing polyacrylamide gel. Size markers are shown in lane 1 and the unextended primer in lane 2. B, the maximum length extension product produced by M-MuLV RT (wt) and RNase H$^-$RT (ssII) were determined as described under “Experimental Procedures” and graphed as a function of time. Data were taken from A (RNA) or otherwise identical reactions performed using the LTR2 non-displacement template (NON) or LTR2 DNA displacement template (DNA) (see Fig. 2A for schematic of templates).
relying on duplex breathing to allow synthesis through double-stranded regions, then it would be expected that differences in the thermostability of RNA-DNA hybrids as compared with duplex DNA would be reflected in the rate of synthesis on the two templates. However, the thermostability of duplex DNA is predicted to be slightly higher than that of hybrid duplex on sequences of random base composition (53–55). Notably, the predicted thermostability of the RNA-DNA hybrid immediately downstream from the primer terminus on the LTR2 template is less than that for duplex DNA of the same sequence, yet on the RNA displacement template RT extends through this region with one-tenth the efficiency observed for DNA displacement synthesis. Thus the data here support and extend the conclusions of Whiting and Champoux2 that while a passive mechanism remains a formal possibility, RT most likely displaces...
DNA and RNA actively using either an SSB-like or helicase-like mechanism. If RT actively participates in strand separation, differences in how RT interacts with the RNA or DNA non-template strands may be responsible for the observed differences between the rates of RNA and DNA displacement synthesis.

In addition to finding that the early stalling observed during RNA displacement on the LTR2 template was sequence-dependent, we found that the initiation of displacement synthesis was also more efficient if a gap rather than a nick preceded the region to be displaced. This finding was surprising since we did not expect the process of initiating displacement to be affected by synthesis occurring upstream of the RNA 5’ end. One possible explanation for this finding is that some minimal length of synthesis, whether it be non-displacement or displacement synthesis, is required to effect a change in the properties of the polymerase that facilitate displacement of the non-template strand. For example, a shift from a distributive to processive mode of synthesis could account for the relatively rapid extension rates observed after an initial stalling, or for the ease of initiating displacement synthesis after extending through a gap. Consistent with this possibility, DeStefano et al. (6) found that the dissociation of RT from a primer-template occurs in a biphasic manner, suggestive of two binding modes for the polymerase with its substrate. Moreover, Whiting and Champoux2 recently found that a distributive to processive transition occurs after the addition of ~10 bases during DNA displacement synthesis.

Enzymatic footprinting of M-MuLV RT by Wohrl et al. (56) suggests that contacts between RT and the template may extend up to 6 bases downstream of the primer terminus (position +6). Thus it is plausible that RT may be required to melt up to 6 bases of the non-template strand before necessary downstream contacts with the template strand can be made. The observation that RT stalls ~6 bases upstream of the 5’ end of the non-template strand on the gapped displacement template may provide tentative support for this hypothesis, although such stalling has not been observed on the other sequences we have tested.

Given that the biologically relevant form of RT contains RNase H activity, it was of interest to measure displacement synthesis by M-MuLV RT under the same conditions used for SSIII. As expected, the rate of synthesis by M-MuLV RT on the RNA displacement template was significantly greater than that catalyzed by SSIII. Since the rates of non-displacement and DNA displacement synthesis were nearly identical for the two enzymes, it seems very probable that RNase H-directed cleavage of the non-template RNA strand was responsible for the increased rate of synthesis by M-MuLV RT. However, despite the ~100-fold molar excess of enzyme over the RNA-DNA template, the rate of synthesis by M-MuLV RT remained less than that on a DNA displacement template; thus the cleavage that occurred over the course of the 20-min incubation was not sufficient to offset the intrinsic difficulty RT appears to have in displacing RNA. Additionally, the median rate of synthesis by M-MuLV RT on the hybrid template showed little increase over that observed with SSIII, despite the slight reduction in the amount of stalling observed during initiation. Extrapolation using the maximum rates (Fig. 5B), however, predicts that on longer hybrid templates the rate of synthesis by M-MuLV RT would surpass that of DNA displacement synthesis. During retroviral replication, there may be sufficient time between minus strand synthesis and the onset of plus strand synthesis for the RNase H activity to reduce the RNA genome to relatively small fragments. Many of these fragments may be short enough to readily dissociate from the DNA; however, if longer fragments remain, then the relatively weak RNA displacement activity of RT could become rate-limiting for the overall process. Alternatively, Miller et al. (57) suggest that RNA displacement synthesis need only progress efficiently through the LTR (to allow for the second jump) as preintegration complexes composed of discontinuous plus strands appear competent to integrate.

Like many SSBs (33), retroviral NC promotes both nucleic acid helix destabilization and strand renaturation (28, 30, 58, 59); thus, it seems reasonable that NC might play the role of an SSB-like accessory factor in reverse transcription. Studies looking at the effects of NC on extension rates, enzyme pausing, and processivity by RT during non-displacement synthesis have yielded contradictory results (39, 41, 44, 45, 60, 61). Similarly, DNA displacement synthesis by HIV-1 RT appears either unaffected (16) or slightly stimulated (42) by NC. To our knowledge, the effect of NC on RNA displacement synthesis has not previously been characterized.

Our titration of NC levels during RNA displacement synthesis shows that a discrete and reproducible transition in the distribution of products occurs when RNA displacement is carried out in the presence of sufficient NC. Such a transition is consistent with previous observations that NC acts stoichiometrically rather than catalytically (27–29, 62). This transition occurred at roughly the same NC:nt ratio required to promote strand annealing in the standard NC annealing assay. The ratio of NC:nt required to promote rapid duplex formation in the annealing assay was several times greater than expected based on the work of Lapadat-Tapolsky et al. (48) using HIV-1 NC, which might reflect either differences in the annealing capacity of the different NC types or decreased activity of our preparation.

Surprisingly we find that stimulation of displacement synthesis by NC is dependent on the presence of the zinc finger domain. Numerous studies looking at the importance of the NC zinc finger motifs have generally concluded that this domain is required for the proper selection and packaging of genomic RNA but that the motif is dispensable for such activities as RNA dimerization, annealing of the tRNA to genomic RNA, strand renaturation, and nonspecific RNA binding (25, 51, 52, 63–65). On the other hand, several studies have provided evidence that the zinc finger domain is critical for viral infectivity beyond the requirement for proper RNA packaging. Point mutations within the zinc finger domains of HIV-1 or M-MuLV NC have been identified that produce virus with only a somewhat reduced RNA content but with a greatly reduced minus strand synthesis capability and no infectivity (66–68). Recent characterization of one such mutant demonstrated that the mutant successfully completes the first template switch but fails to synthesize full-length minus strand DNA (69). The failure of this mutant to complete reverse transcription cannot be explained by the loss of NC annealing or RNA dimerization activities since these activities have been shown to be independent of the zinc finger (51, 63). It is possible that this mutant, like the NCdd zinc finger mutant in results presented here, fails to facilitate RNA and DNA displacement synthesis.

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