Single Unpaired Nucleotides Facilitate HIV-1 Reverse Transcriptase Displacement Synthesis through Duplex RNA

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During reverse transcription of viral RNA, HIV-1 reverse transcriptase (RT) encounters RNA stem-loop structures that require displacement synthesis activity in which RT disrupts the RNA helix to access the template strand. A primer extension assay was developed to assess HIV-1 RT RNA displacement synthesis activity in vitro. Initial results revealed that HIV-1 RT performs only limited amounts of RNA displacement through long stretches of RNA duplex, with the majority of synthesis stalling at sequence-dependent pause positions. DNA displacement synthesis through the same sequence, however, proceeded rapidly to the end of the template. The RNA folding algorithm mfold indicated that the presence of an unpaired nucleotide, or “bulge,” along the RNA duplex would promote helix melting ahead of the DNA primer terminus to create a small gap of non-displacement synthesis. Primer extension assays using substrates possessing single-nucleotide bulges in the nontemplate strand near pause sites resulted in diminished pausing at positions within the predicted melted region. Surprisingly, the bulges also reduced pausing distal to the bulge at positions that are expected to remain base-paired. Further analysis revealed that stalling during RNA displacement synthesis results from the displaced RNA re-annealing to the template strand thus forcing the primer terminus to become unpaired and, therefore, not extendable. Introduction of a bulge facilitates displacement synthesis through distal regions by increasing RT processivity in the vicinity of a bulge and reducing the impact of branch migration on primer extension.

HIV-1 reverse transcriptase (RT) is a heterodimer consisting of 66- and 51-kDa subunits and is the viral polymerase necessary to convert the positive-sense, single-stranded RNA genome to double-stranded DNA for subsequent integration into the host cell genome. HIV-1 RT is capable of synthesizing DNA using RNA and DNA in all possible primer-template combinations. Reverse transcriptases in general possess two other activities in addition to the polymerase function. The first is a ribonuclease H (RNase H) activity, which hydrolyzes RNA in RNA-DNA hybrid duplexes. The second is a displacement activity, which allows the enzyme to synthesize DNA through regions of duplex nucleic acid (2–4).

Not all DNA polymerases can perform displacement synthesis and often require accessory factors in the form of helicases and single-stranded DNA-binding proteins (5-9). Although retroviral nucleocapsid can enhance displacement synthesis, retroviral RTs have an intrinsic ability to synthesize DNA through duplex structures in the absence of other factors (10–16). In vitro primer extension assays show that the RTs from Moloney murine leukemia virus (15, 16), feline immunodeficiency virus (17, 18), avian myeloblastosis virus (19, 20), and HIV-1 (2–4) all possess strand displacement activity. Importantly, mutating the Phe-61 residue in HIV-1 RT revealed that displacement activity is functionally separable from primer-template binding and the polymerase activity, as some of the mutant proteins displaced DNA less efficiently than the wild type, whereas processivity remained unchanged (21).

During reverse transcription, RTs encounter three types of duplex nucleic acid that require displacement synthesis. First, to complete the formation of the long terminal repeat after the second strand transfer, RTs must displace several hundred consecutive DNA bp. In vitro displacement assays have shown that both Moloney murine leukemia virus and HIV-1 RT are capable of extensive and processive DNA displacement in the absence of accessory factors, thus fulfilling this requirement (4, 15, 16). Moreover, earlier studies with avian retroviruses suggested that plus-sense DNA is polymerized discontinuously, generating single-stranded flaps caused by upstream primer extension displacing downstream fragments (22, 23). A similar phenomenon generates a single-stranded flap in the region of the central polyuridine tract of HIV-1 (24, 25).

The second duplex structure is RNA annealed to a DNA template that also occurs during plus-sense DNA synthesis. As RT synthesizes minus-sense strand DNA, the RNase H activity hydrolyzes the template RNA. This cleavage, however, is not complete, and some RNA likely remains annealed to the newly synthesized DNA (26, 27). Further RNase H digestion can remove these remnants, but both HIV-1 RT and Moloney murine leukemia virus RT are capable of displacing them in the absence of a functional RNase H domain (19, 27).

The third type of duplex nucleic acid is RNA annealed to an RNA template. The single-stranded nature of retroviral genomes allows for the formation of stem-loop structures that create segments of duplex RNA. The HIV-1 genome, for example, possesses stem-loops in the R/U5 regions in the form of the trans-activation response (TAR) element and the poly(A) signal (1). Moreover, the tRNA(Lys3)/primer binding site interaction creates an 18-bp RNA duplex that HIV-1 RT encounters just prior to the second strand transfer. For complete minus-sense DNA synthesis, RT must polymerize efficiently through these structures.

The kinetics of HIV-1 RT RNA displacement synthesis have...
been addressing the hairpin structures with stems of 8 or 12 contiguous RNA bp derived from the HIV-1 sequence (28, 29). Other investigators have assessed synthesis through larger double-stranded structures of the HIV-1 genome, including 17 contiguous bp within the poly(A) signal (13, 14). In the course of comparing the processivity of avian myeloblastic virus and the arthropod retro-transposable element R2 RTs on single-stranded DNA templates, Bilbo and Eckbush (30) also compared how these two enzymes displace through 117 contiguous bp of RNA (30). The results indicated that avian myeloblastic virus RT displaced very few nucleotides, with significant accumulation of pause products at early template positions. One common feature to these systems is that RT pauses at specific positions during RNA displacement synthesis. AU/AU-G/C, for example, is a loose consensus sequence correlated with RT pausing, especially if the template residues are base paired (31). Pausing has also been correlated with the overall thermodynamic stability of stem-loop structures, suggesting that synthesis through double-stranded RNA most likely relies on duplex breathing ahead of the primer terminus (29, 32).

To determine the full extent to which HIV-1 RT can displace RNA in the absence of accessory factors and how this relates to a mechanism of RNA displacement synthesis, in vitro primer extension assays were developed to assess polymerization through long stretches of RNA duplex. Initial results indicated that this process was inefficient compared with both nonden-placement and DNA displacement synthesis. However, interrupting contiguous RNA duplex by introducing single-nucleotide bulges into the nontemplate strand decreased pausing in the region surrounding the bulge nucleotide. The effect of the unpaired nucleotide on RNA displacement synthesis was 2-fold. First, RT processivity increases in the vicinity of the bulge, even if nearby pause sites are predicted to remain base paired. The second involves branch migration, in which the displaced RNA strand anneals back to the RNA template, re-

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained as follows: HIV-1 RT was from Worthington. Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, and Vent polymerase for PCR were from New England Biolabs. pGEM9zf(+) and the T7 or SP6 RiboMax transcription kits were from Promega. [γ-32P]ATP was from PerkinElmer Life Sciences. Pfu DNA polymerase for site-directed mutagenesis was from Stratagene. Reagents used for RNA solutions were from Ambion. S1 nucleases was from Invitrogen. DNA oligonucleotides were from Invitrogen or Qiagen.

Plasmid Constructs—For construction of RntA and the RNA template (LT) containing pGEM9zf(+) was linearized with SacI, 5'-overhangs were filled in with T4 DNA polymerase, and then this vector was treated with NsiI. For RntA, nucleotides 791–1093 from the HIV-1 HXB2 clone were amplified using the following primers: SmaT7F (5'-overhangs were ligated with SacI, T7 DNA polymerase, and EcoRI-treated pGEM9zf(+)). The short nontemplate RNA (5'-RntA) was generated by PCR mutagenesis to introduce a SmaI site 40 bp from the SP6 transcription start site. The short DNA template (sDT-1) and nontemplate (sDntA) strands were ordered from Invitrogen and gel purified. All clones were verified by sequencing the entire RNA template region.

RNA Folding—All RNA folding was performed using the algorithm of Zuker (www.bioinfo.rpi.edu/zukerm/rna/). Changes in nontemplate strand folding during RNA displacement were assessed by first folding a hairpin with 45 contiguous RNA bp representing the nontemplate strand annealed to the template strand. The 3'-end of the template strand was extended by 27 nucleotides and forced to remain unpaired to represent labeled DNA oligonucleotide annealed to the template. This strategy was based on the assumption that the presence of the primer would exclude these nucleotides from interacting with other RNA bases. Successively forcing the next template base to be single-stranded was used to simulate displacement synthesis. This interrupted the RNA bp and allowed the nontemplate base to fold into any new structures. With each new structure the most thermodynamically stable one was considered to be the dominant theoretical structure.

In Vitro Synthesis and Purification of RNA Substrates—RntA and derivatives were linearized with SmaI, and LT-1 was linearized with BglII. RNA was generated from either a T7 or SP6 promoter using the RiboMax in vitro transcription kit. After in vitro transcription, the products were phenol/chloroform extracted and precipitated with 2.5 volumes of ethanol. The RNA was collected by centrifugation, washed once with 70% ethanol, and dried. RNA pellets were dissolved in 15 μl of 10 mM Tris, pH 8, 1 mM EDTA (TE) and 45 μl of formamide/EDTA, boiled for 3 min, and then purified by denaturing PAGE. The full-length RNA was visualized by UV shadowing, excised, and eluted for at least 18 h in a 1:1 v/v mixture of TE and acidic phenol/chloroform. The RNA was then centrifuged and the aqueous phase removed. The aqueous phase was extracted once with chloroform, and the RNA was precipitated with an equal volume of isopropanol alcohol. The mixture was centrifuged, washed twice with 70% ethanol, and dried. The pellet was dissolved in 50 μl of TE and run through a Chromaspin DEPC H₂O C-30 size exclusion, spin column (BD Biosciences). Tris, pH 8.0, and EDTA were added back to 10 mM and 1 mM, respectively, and the volume was brought to 50 μl. Concentration of the RNA was determined by UV absorbance at 260 nm. For RntB and RntC nontemplate RNAs, the RntA RNA was synthesized and then annealed to the DNA oligonucleotides 5'-SP6DH (5'-CCAAGGAAGCTTTAGACATGCA-3') or SP6DH (5'-CCACCTCTATGTGTGTCA-3'), respectively. HIV-1 RT was then added in the absence of nucleotides so that the RNA H activity would cleave the template RNA. The cleaved RNA was purified in the same manner as above, and the 5'-ends of the RNA were confirmed by primer extension with Superscript 2 (Invitrogen) compared with a sequencing ladder generated using the same primer on a DNA template.

Primer Extension Assays through RNA Template/Nontemplate Structures—For primer extension from a nick through RntA, RntB, RntC, sRntA, or sDntA substrates, the following primers were used: LTNK 5'-ACTTTTGTTTTCTCCTTCCTATCTTT-3' (5'-AAATGCGAGCTTAAAGCTTCC-3'), LtB 5'-CTCTT-GAATTGATCTAGTTAAG--3', and LtC 5'-ATCTCCTCTCCTGCTATGCT-3'. Head start primers LtnF5S and LtnR7S consisted of LTNK plus 5' and 7' complementary nucleotides added at the 3'-end. Each RNA primer was gel purified and 5'-end labeled in the presence of [γ-32P]ATP by polynucleotide kinase. The RNA or DNA template was annealed to end-labeled DNA primer in the presence of 100 mM Tris, pH 8.0, and 100 mM KCl at a 2:1 molarity ratio by heating to 95 °C for 5 min and cooling to 37 °C at a rate of 0.02 °C/s using a NanoDrop thermocycler (Savant). Nontemplate RNAs were annealed to the template RNA or DNA at a 3:1 molar ratio at 37 °C for 5 min. DTT, MgCl₂, and 7 pmol of HIV-1 RT were added, and the mixture was incubated further at 37 °C for 30 min. Primer extensions were then started by the addition of dNTPs. Final concentrations were: 50 mM Tris, pH 8.0, 50 mM KCl, 10 mM DTT, 5 mM MgCl₂, and 0.1 μl of HIV-1 RT. At each time point, 2.5 μl of the reaction was transferred to 12 μl of loading buffer (96% formamide and 20 mM EDTA). Extension products were separated by denaturing PAGE. Dried gels were visualized using a Storm PhosphoImager and analyzed using ImageQuant software.
S1 Nuclease Sensitivity Assay—The 5’-end-labeled DNA primer was annealed to the RNA template at a molar ratio of 1:2 by heating to 95 °C and cooling to 37 °C at a rate of 0.02 °C/s in the presence of 100 mM Tris, pH 8.0, and 100 mM KCl. A nontemplate RNA (or equal volume of TE as a storage buffer (10 mM potassium phosphate, pH 7.4, 1 mM DTT, and 20% glycerol (v/v)) were then added as described for the primer extension assay, and reactions were incubated for an additional 5 min. DTT, MgCl₂, dNTPs, and HIV-1 RT storage buffer (10 mM potassium phosphate, pH 7.4, 1 mM DTT, and 20% glycerol (v/v)) were then added as described for the primer extension assays, and reactions were incubated for an additional 5 min. 8 µl of this mixture was then transferred to 12 µl of S1 nuclease mix (2 µl of 10× buffer (300 mM sodium acetate, pH 4.6, 10 mM zinc acetate, 50% v/v glycerol), 2 µl of 1.3 units/µl S1 nuclease, and 8 µl of H₂O). The final pH of the solution was between 7.0 and 8.0. 3-µl aliquots were removed at each time point and added to 12 µl of loading buffer. Samples were analyzed as described above. This process was repeated in the absence of template RNA for the primer cleavage control.

Single Round Primer Extension (“Trap”) Assay—Labeled primer and RNA template were annealed at a 1:1 ratio (0.5 pmol each) using conditions as described above. Nontemplate RNA was then annealed at 37 °C for 5 min at a ratio of 3:1 nontemplate:template. MgCl₂, DTT, and 2.5 µM of HIV-1 RT (final concentration as above) were then added except in the case of the pre-trap, in which a mixture of 200 µg of heparin and 30 µm of an unlabeled DNA-RNA primer-template substrate was added first. Reactions were incubated for 5 min at 37 °C. A mixture of the heparin, unlabeled substrate, and dNTPs was then added to each tube to start the reactions except for the pre-trap in which only dNTPs were added.

RESULTS
HIV-1 RT Performs Limited RNA Displacement Synthesis on a Long Duplex RNA—Previous work demonstrated the ability of RT to carry out DNA displacement synthesis through duplex DNA that is hundreds of bp in length (4, 15). A similar system was employed for the initial studies to assess the ability of HIV-1 RT to perform displacement synthesis through duplex RNA. We designed an in vitro primer extension assay in which a 412-nucleotide RNA template was annealed to three different 5’-end-labeled DNA primers, LTNa, B, and C (Fig. 1A). Three complementary, nontemplate RNA strands, RntA, RntB, and RntC, were then annealed directly downstream from the respective primers. Extension of each labeled primer required initiation at a nick followed by displacement synthesis through duplex RNA. HIV-1 RT was added to substrates, extension reactions were initiated by the addition of dNTPs, and products were separated by denaturing PAGE. Fig. 1B shows a time course of nondisplacement and displacement synthesis for each of the structures depicted in Fig. 1A. In each case, the presence of an annealed nontemplate strand significantly reduced the amount of full-length product formed compared with nondisplacement synthesis. For example, within the 30-min time frame of extension through RntA, only a small fraction of primer was extended beyond position +20, and there was accumulation of pause products at +2, +5, +6 and +7 (Fig. 1B). None of these early pause sites was observed in the absence of a nontemplate strand, suggesting that contiguous duplex RNA prevents efficient extension. Similarly strong pausing was seen with LTNb and LTNc, al-
though longer extension products were also observed (Fig. 1B).

To compare the ability of HIV-1 RT to synthesize through duplex RNA versus duplex DNA, extension through a short version of RntA (sRntA) was compared with extension through duplex DNA (Fig. 2). The RNA substrate is 10 bp longer than the DNA duplex, which accounts for the longer full-length product observed after denaturing PAGE. In the case of DNA duplex there was only a slight delay in synthesis of the 61-nucleotide full-length product, whereas the RNA duplex gave results virtually identical to those in Fig. 1B. These results indicate that DNA displacement is more efficient compared with RNA displacement under identical reaction conditions. We also found that shifting the position of the DNA primer back from the start of the RNA duplex to create a gap had no effect on the pause pattern or distance displaced into the RNA duplex (data not shown).

Single, Unpaired Nucleotides in the Nontemplate Strand Are Predicted to Facilitate RNA Displacement Synthesis—The initial primer extension studies discussed above demonstrated that HIV-1 RT has limited capacity to perform extensive RNA displacement synthesis through long stretches of contiguous RNA bp. Duplex structures within the HIV-1 viral genome, however, are typically interrupted by unpaired nucleotides or mismatches. The TAR element and polyadenylation signal are, however, are typically interrupted by unpaired nucleotides or mismatches. The TAR element and polyadenylation signal are typically interrupted by unpaired nucleotides or mismatches. The TAR element and polyadenylation signal are typically interrupted by unpaired nucleotides or mismatches. The TAR element and polyadenylation signal are typically interrupted by unpaired nucleotides or mismatches. The TAR element and polyadenylation signal are typically interrupted by unpaired nucleotides or mismatches. 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can facilitate HIV-1 RT RNA displacement synthesis through duplex RNA.

Greater Processivity Occurs in the Region of an Unpaired Nucleotide—To explore further the effects of the bulge nucleotide on RNA displacement synthesis, primer extension assays were repeated in the presence of heparin plus an unlabeled DNA primer annealed to an RNA template to limit polymerization to a single enzyme-binding event. A pre-trap control was included to show that there was sufficient trap to sequester all of the added polymerase (Fig. 5). For RntA and OH-1, strong pausing was again observed at +2, +5, +6, and +7. Processivity through the +2 site was increased for the 1.5B and 2.5B substrates, but it was not until the 3.5B bulge that the +2 pause site disappeared completely. The pausing observed at +2 for the 1.5B and 2.5B suggests that RT dissociation may be the result of inefficient incorporation of the +3 nucleotide when the +3 position is duplex. The +3 position is predicted to be single-stranded in 3.5B, 4.5B, and 5.5B. No pausing is observed at +2 for 3.5B and 4.5B, but the +2 pause reappears in 5.5B, which suggests that the predicted helix melting may not occur until incorporation of the 3rd nucleotide in 5.5B rather than the predicted 2nd nucleotide. Regardless, more processive synthesis is observed through the +5, +6, and +7 pause sites for substrates 5.5B, 6.5B, and 7.5B.

Similar to the distributive polymerization experiment shown in Fig. 4, as the bulge proceeds from 8.5B to 10.5B, the +5, +6, and +7 pause sites successively reappear, while at the same time, the +11 and +12 pause sites diminish in relative strength (Fig. 5, 8.5B, 9.5B, and 10.5B). Moreover, no significant pausing was observed at any of the positions discussed above when trap experiments were performed in the absence of a nontemplate strand (data not shown).

A Head Start in RNA Displacement Synthesis Does Not Affect HIV-1 RT Pausing within Duplex RNA—The trap results above indicate that an unpaired nucleotide present in the nontemplate strand of an RNA helix can increase HIV-1 RT processivity through 2 bp on either side of the bulge residue. The melting phenomenon predicted by mfold can account for decreased pausing at the primer-proximal bp by causing RT to switch to the nondisplacement synthesis mode. However, helix melting cannot explain a decrease in pausing at bp distal to the melted region as was observed at template positions +5, +6, and +7 in substrates such as 1.5B and 3.5B. This leads to the question of why, when HIV-1 RT reaches template position +7, can synthesis continue to +8 and beyond when a bulge is present yet in the context of RntA, extension beyond +7 is inefficient.

To address this question, we first tested whether DNA synthesis through the bulge region itself was required for the observed decrease in pausing. Two primers were designed to give a head start in RNA displacement synthesis (Fig. 6A). LTnP5 and LTnP7 consist of the LTnA primer used in the original primer extension assays (Figs. 1 and 3) plus 5 or 7 nucleotides added to the 3′-end, respectively. Extension of LTnP5 on bulge substrates simulates duplex melting and synthesis up to the last unpaired template base, whereas extension of LTnP7 simulates duplex melting and synthesis 2 nucleotides into the RNA duplex beyond the bulge residue. The two labeled head start primers were annealed to the template RNA first, followed by low temperature annealing of either the RntA (no bulge) or 5.5B (bulge) nontemplate strands. Annealing efficiency was similar for both as indicated by native PAGE (data not shown). Fig. 6B shows the results of the primer extension assay for these substrates. When a nontemplate strand lacking a bulge was annealed to template with either LTnP5 or LTnP7, primer extension was minimal and comparable with the pausing observed in Fig. 1B. When the bulge residue was present, however, both primers were extended to the next set of pause sites (+11, +12, and +17; see Fig. 6B). Without a nontemplate strand, both LTnP5 and LTnP7 are rapidly extended to the end of the RNA template (data not shown).

Branch Migration Occurs in the Presence or Absence of a Bulge Residue, but Extension Is Favored by the Extra Nucleotide—The results in Fig. 6B were surprising because the only difference between these substrates is the presence of 1 extra nucleotide in the already displaced flap. Any effect of a bulge residue should not be a factor in this particular situation because the primer is essentially beyond the +5/+6 template positions. To explain the data, we hypothesized that the process of branch migration might be occurring, such that the displaced RNA re-anneals back onto the template strand when HIV-1 RT dissociates at a pause site. If this were to occur, then the 3′-end of the nascent DNA would be pushed off the tem-
plate and could no longer serve as a primer for polymerization. Accordingly, the presence of a single, unpaired nucleotide in the nontemplate RNA in the region overlapping the nascent DNA would perhaps destabilize the RNA helix sufficiently to favor the DNA remaining annealed to the template.

To address this hypothesis, we designed an S1 nuclease sensitivity assay. In the absence of enzyme, radioactively labeled LTnP5 and LTnP7 primers were annealed to template RNA followed by low temperature annealing of either RntA (no bulge) or 5.5B (bulge) (see "Experimental Procedures"). If branch migration occurred, the 5' end of the nontemplate RNA would replace the annealed 3' end of the DNA and render the DNA primer sensitive to the single-strand-specific S1 nuclease. Fig. 7A shows the results of this assay for the labeled LTnP7 primer. When annealed to the template strand, LTnP7 was not sensitive to S1 nuclease (Fig. 7A, lanes 6–9), whereas LTnP7 alone was completely degraded under these conditions (Fig. 7A, lanes 1–5). When either RntA or 5.5B was annealed, however, the primer was cleaved by S1 nuclease within the 7 3' nucleotides that overlap with the flap of the nontemplate strand. Similar results were found using LTnP5 with cleavage only occurring within the 5 3' nucleotide overlapping region (data not shown). Although these results confirmed that branch migration is possible, the amount of cleavage observed for substrates with or without a bulge was not significantly different within the time scale of these experiments (Fig. 7B). The primer extension results, however, indicate that when a bulge is present the primer terminus must remain annealed long enough for nucleotide addition to occur.

In light of this apparent contradiction, rather than preventing branch migration, a bulge residue may somehow destabilize the RNA duplex enough to allow the nascent DNA to re-anneal more readily to the template once branch migration has occurred. If this were the case then we would expect that an extra, unpaired nucleotide would only affect LTnP7 extension if it were within the 7 nucleotides overlapping with the nontemplate RNA. We therefore repeated the LTnP7 primer extension with all the bulge substrates (Fig. 8A). As before, RntA demonstrated no extension. Adding 1 extra nucleotide at the 5'-end of the nontemplate strand also resulted in virtually no primer extension. Once the bulge residue is within the overlapping 7-nucleotide region, however, LTnP7 is extended more effi-
ciently to the next set of pause products. Extension tapers off from 6.5B to 7.5B and is virtually absent in 8.5B to 10.5B (Fig. 8A). Importantly, if LTnP7 was annealed after the nontemplate RNA was annealed the same results were obtained (Fig. 8B). As the schematic indicates, by annealing in this order, the 7 nucleotides at the 3’-end of LTnP7 should initially be unannealed. The only way to extend the primer is if the 3’-end of LTnP7 replaces the 5’-end of the nontemplate RNA by branch migration. These experiments demonstrate that an increase in primer extension only occurs when the bulge residue is within the overlapping region and confirms that an unpaired nucleotide in the nontemplate RNA promotes the conformation where the DNA primer terminus is annealed to the template.

**DISCUSSION**

Results presented here demonstrate that RNA displacement is much less efficient than DNA displacement through identical sequences. In our system, very strong pause sites can be observed at early positions within RNA duplex. Extension through RntA, for example, showed significant accumulation of pause intermediates within the first 11 bp, whereas very little pausing was observed during DNA displacement synthesis (Figs. 1 and 2). Although HIV-1 genomic secondary structures such as the TAR element consist of duplex stems greater than 11 bp long, primer extension is rapid through the stem in vitro (34). One difference between these genomic sequences and our model substrates lacking bulges (see Fig. 1) is that contiguous base pairing in the former is interrupted by single or multiple unpaired nucleotides (bulges) in the stem sequences. We designed and tested different substrates to determine whether the presence of the unpaired nucleotides affected RNA displacement synthesis. Indeed, these bulges appear to have two consequences in relation to polymerase pausing within regions of double-stranded RNA: increased enzyme processivity and attenuation of branch migration. These effects are illustrated in Fig. 9 and discussed below.

**Increased HIV-1 RT Processivity**—The insertion of an unpaired nucleotide decreases pausing in the vicinity of the bulge by increasing processive synthesis. This is observed regardless of which base creates the bulge or whether the bulge is in the template or nontemplate strand (data not shown). The data in Fig. 5 show that HIV-1 RT processivity increases through approximately 2 bp both 5’ and 3’ of the bulge residue. A simple explanation for these observations is that the template region surrounding the bulge is unpaired from the start of the primer extension assay, which would create conditions for more processive nondisplacement synthesis. This might result from the nontemplate strand folding into a secondary structure as RT progresses into the RNA helix (32). Alternatively, the unpaired base might destabilize the duplex to such an extent that base pairing is energetically unfavorable. However, three lines of evidence contradict this notion. The first is that the mfold algorithm (33) predicted that prior to synthesis the 5’-RNA segment would anneal to the template strand with the only alteration being the bulged residue except for constructs 1.5B and 2.5B (Fig. 4A). Moreover, no secondary structures that could disrupt the helix were predicted to form during the process of displacement synthesis. Second is a crystal structure of an RNA duplex with 9 bp interrupted by a single cytosine bulge, which revealed that despite a 10° bend in the A-form helix and a slight widening of the minor groove on either side of the bulged residue, significant alterations in base pairing in the vicinity of the bulge were not observed (35). The third line of evidence is that the first 7 nucleotides on the 5’-ends of both RntA (no bulge) and 5.5B (bulge) nontemplate RNA strands compete equally with an overlapping DNA primer for annealing to the RNA template (Fig. 7), suggesting that the nontemplate strand of 5.5B is not otherwise unpaired.

RNA folding predictions did indicate that duplex melting ahead of the primer terminus could account for increased processivity through the 5’-bp. For example, mfold predicted that after displacement of 2 nucleotides in substrate 5.5B, the RNA duplex would melt up to template position +5, whereas bp at +6 and beyond would remain intact (Fig. 3). This creates nondisplacement synthesis conditions from template positions +3 to +5 and, because nondisplacement synthesis is more processive compared with displacement synthesis (15, 16, 27), accounts for the increased processivity up to +5.

The mechanism by which bp 3’ to the bulge are more readily displaced even though they are predicted to remain intact after duplex melting, however, is problematic. Perhaps after a short stretch of nondisplacement synthesis following the bulge-induced melting the more processive mode is sustained for several nucleotides of displacement synthesis. This explanation is unlikely because increasing the distance between the DNA primer terminus and the 5’-end of an RNA nontemplate strand to provide a short gap had no effect on the pause pattern or distance displaced into the RNA duplex (data not shown). However, shifting the primer position relative to the start of RNA duplex does not provide a nontemplate strand flap like the melting phenomenon (Fig. 3). It could be argued, therefore, that the presence of a flap enables processive synthesis for a few nucleotides into RNA duplex. When there is no bulge nu-
cleotide present, however, any enhancing effects of a flap on further processive synthesis are complicated by the branch migration phenomenon shown in Figs. 6–8.

**Attenuation of Branch Migration**—Branch migration in this context is the process by which a displaced RNA strand can re-anneal to the RNA template when HIV-1 RT dissociates at a pause site, resulting in the unpairing of the 3'-end of the nascent DNA from the template. Branch migration has previously been suggested to occur during DNA displacement synthesis (36), and unpaired nucleotides have also been shown to affect branch migration in the context of recombination. For instance, Panyutin and Hsieh (37) demonstrated that the presence of a mismatch could bias the direction of movement of a Holliday junction.

Our results suggest that branch migration allows the displaced RNA to disrupt primer extension and create strong pause sites. This effect, however, is ameliorated by the presence of a single unpaired nucleotide within the overlapping regions of the nascent DNA and non-template RNA. Competition for annealing to an RNA strand between DNA and RNA is...
unique to RNA displacement synthesis during reverse transcription. The effects of branch migration on DNA displacement synthesis are probably minimal because annealing of the nascent DNA or nontemplate DNA has virtually identical thermodynamic stability. On the other hand, the RNA-RNA duplex is more stable than an RNA-DNA duplex (38, 39) and therefore once the DNA is unpaired initially, it is energetically unfavorable for the nascent DNA to re-anneal. This is consistent with our folding predictions demonstrating disruption of the RNA helix by an unpaired nucleotide during displacement synthesis (Fig. 3). Thus for RNA displacement synthesis, a bulge nucleotide in the nontemplate strand enables the nascent DNA to re-anneal to the template more efficiently after branch migration occurs.

**Potential Effects on in Vitro Strand Transfer**—The current model of strand transfer involves a “dock” and “lock” mechanism driven by RT pausing on RNA templates (40, 41). Briefly, the process begins when a pause site interrupts DNA polymerization on a “donor” template. This pausing allows the RNase H activity of RT to degrade the RNA template behind the primer terminus. The nascent DNA is then available to anneal to a complementary “acceptor” strand (docking). The remainder of the nascent DNA transfers onto the acceptor strand by branch migration (locking). The increased processivity and decreased pausing observed in the vicinity of a bulge could decrease the amount of strand transfer by preventing the RNase H activity from “catching up” to the DNA 3’-end (41). The attenuation of branch migration in the presence of a bulge nucleotide could affect both docking and locking processes. At a strong pause site within duplex RNA, branch migration of the
displaced RNA could push the 3′-end of the nascent DNA off the RNA template, stalling synthesis and making the 3′-end of the DNA readily available for locking onto the acceptor template. The unpaired primer terminus could also enhance DNA-to-DNA strand transfer or in the “primer terminus switch” model explaining the TAR mediated strand transfer reaction (41, 42). When an unpaired base is present, however, the DNA more readily re-annales back to the template (Fig. 8B), enabling continued RT polymerization. This would not only prevent the docking from catching up to the 3′-end of the DNA, but it would also move the lock site further along the template as described previously (41).

The potential effects of single nucleotide bulges on strand transfer and, by extension recombination, prompted us to speculate that in the course of viral evolution the destabilizing effects of bulges may have been a selected feature to ensure a high rate of reverse transcription through RNA duplexes and to decrease the probability of losing stem-loops as a consequence of recombination. Clearly, bulges are important as part of the overall tertiary structure of stem-loops and can be involved in interactions with other factors critical for the viral life cycle. Unpaired nucleotides along the HIV-1 TAR stem, for example, are necessary for binding of the viral Tat protein as well as a host cell-derived protein (43, 44). However, assuming that secondary structures were present in the viral genome prior to gaining a protein interaction function, then one potential selective pressure would be how efficiently the structure is replicated during reverse transcription. If the duplex portion of a stem-loop has a strong pause site then the probability of losing the structure because of aborted reverse transcription or recombination, increases. On the other hand, the presence of a bulge in the vicinity of a strong pause site would increase processive synthesis through the structure while decreasing the probability of recombination and would ultimately facilitate the maintenance of a stem-loop structure within the viral genome. As a result, those structures that were maintained in the genome could have provided a structural pool from which RNA-protein interactions evolved.

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