Temporal Coordination between Initiation of HIV (+)-Strand DNA Synthesis and Primer Removal*

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In this study, we have analyzed the interdependence between the polymerase and RNase H active sites of human immunodeficiency virus-1 reverse transcriptase (RT) using an in vitro system that closely mimics the initiation of (+)-strand DNA synthesis. Time course experiments show that RT pauses after addition of the 12th DNA residue, and at this stage the RNase H activity starts to cleave the RNA primer from newly synthesized DNA. Comparison of cleavage profiles obtained with 3' and 5'-end-labeled primer strands indicates that RT now translocates in the opposite direction, i.e. in the 5' direction of the RNA strand. DNA synthesis resumes again in the 3' direction, after the RNA-DNA junction was efficiently cleaved. Moreover, we further characterized complexes generated before, during, and after position +12, by treating these with Fe2++ to localize the RNase H active site on the DNA template. Initially, when RT binds the RNA/DNA substrate, oxidative strand breaks were observed at a distance of 18 base pairs upstream from the primer terminus, whereas 17 base pairs were observed at later stages when the enzyme binds more and more DNA/DNA. These data show that the initiation of (+)-strand synthesis is accompanied by a conformational change of the polymerase-competent complex.

Retroviral RTs are multifunctional enzymes possessing RNA- and DNA-dependent polymerase activities and a ribonuclease H (RNase H) activity that degrades the RNA strand of RNA/DNA hybrids (1, 2). Like other retroviruses, human immunodeficiency virus type 1 (HIV-1) uses a cellular tRNA primer to initiate reverse transcription from a complementary primer-binding site (PBS) near the 5'-end of the viral RNA (3–6). Despite changes of binding and kinetic properties, observed concomitant with synthesis of the first DNA strand (7), i.e. (+)-strand DNA, complexes with the initially bound RNA/RNA duplex and the newly synthesized DNA/RNA substrates share common features. RNase H cleavages on the RNA strand of DNA/RNA primer/template combinations occur at a constant distance of 18 bp upstream of the nascent primer terminus (8, 9). Analogously, RNase H-induced cleavages within the tRNA/RNA duplex, designated as RNase H* activity (10), were observed at the same distance from the 3'-end of the primer, although these cuts are restricted to stalled complexes (11). Together, these data provide strong evidence that RT binds to both RNA/RNA and DNA/RNA substrates with the same orientation, and the number of bp between the two active sites is 18 in each case.

RT-DNA/DNA complexes, which are generated during (+)-strand synthesis, have been relatively well characterized (12–15). The crystal structure of HIV-1 RT complexed to an 18-base primer/19-base template DNA homoduplex (12) suggests that the first 7 DNA/DNA base pairs near the polymerase active site adopt an A-type conformation, whereas the region further upstream is in the preferred B-conformation, both structurally distinct segments being separated by a kink.

Little information is currently available regarding the interaction between RT and the RNA/DNA primer/template combination that is initially bound during (+)-strand synthesis. A short segment near the 3'-end of viral genomic RNA, termed the polypurine tract (PPT), is resistant to RNase H degradation and, unlike the rest of the genomic RNA, remains intact during synthesis of the (-)-strand DNA. The PPT fragment then serves as a primer for (+)-strand polymerization, whereas the (-)-strand DNA is used as a template to guide synthesis. Later after initiation, the RNA primer is removed by RNase H cuts at the DNA-RNA junction and adjacent positions (16, 17).

Here, we demonstrate that the RNA primer is cleaved precisely after the 12th DNA residue has been incorporated. This is an unexpected result, since the relative positions of the polymerase and RNase H active sites would not allow DNA synthesis and RNase H degradation to occur at the same time on the same strand. In contrast, the spatial relationship between both active sites facilitates temporally coordinated activities on opposite strands, in a distance of about 18 base pairs, when RT is complexed with DNA/RNA primer/templats (9).

We now show that RT pauses after addition of the 12th DNA residue during the initiation of (+)-strand synthesis. At this point, another RT molecule (not the one that accomplishes DNA synthesis) binds the substrate in an RNase H-competent binding mode to cleave the RNA primer. The directionality of the latter reaction, i.e. in 5' direction with respect to the RNA strand, distinguishes this binding mode from the polymerase-competent complex. In the polymerase-competent mode, RT binds its RNA/DNA substrate in the same orientation as that described for DNA/DNA, RNA/RNA, and DNA/RNA primer/templates. This has been shown by treating stalled complexes with Fe2++, which allowed us to localize the RNase H active site.

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¶ The abbreviations used are: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; PBS, primer-binding site; PPT, polypurine tract; nt, nucleotides; bp, base pairs; dNTP, 3'-deoxyribonucleoside triphosphate; ddNTP, 2',3'-dideoxyribonucleoside triphosphate; ds, double-stranded.
on the DNA template (15). Together, our data provide a detailed model for the early steps of (+)-strand DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Nucleic Acids and HIV-1 RT—Oligonucleotides** used in this study were derived from the polypurine tract located near the 3′-end of the HIV-1 genome (HXB-2 isolate). DNA oligonucleotides as well as RNA and chimeric DNA-RNA primers were synthesized on an Applied Biosystems 392/8 synthesizer using the standard phosphoramidite method, followed by purification on 12% polyacrylamide, 7 M urea gels containing 50 mM Trisborate, pH 8.0, 1 mM EDTA, 5′-End labeling of oligonucleotides (DNA, RNA, or chimera) was conducted with [γ-32P]ATP and T4 polynucleotide kinase; and 3′-end labeling of the DNA template was performed with [α-32P]dATP using terminal transferase (Boehringer Mannheim) according to the manufacturer’s recommendation. The chimeric 3′-end-labeled primer (3D-17R) was generated by extending the pure RNA PPT-primer (17R) with HIV-1 RT (50 nM) in a buffer containing 50 mM Tris-HCl, pH 7.8, and 50 mM NaCl. Polymerization and RNase H degradation was initiated with Mg2+, 1 mM EDTA. 5

**Results**

**FIG. 1. RNA/DNA primer/template combination used to study early events during the initiation of (+)-strand synthesis.** We devised a DNA template (T-PPT 57D) and a chimeric DNA-RNA primer (Pr + PPT 3D-17R) that serve as substrates in this in vitro study. The chimeric primer contains 17 RNA nt at its 5′-end (bold) and three DNA residues at its 3′-end that would have been incorporated during the first three steps of DNA synthesis. Thus, incorporation of 1, 3, 8, and 9 nt to the chimeric DNA-RNA primer yields complexes termed registers 4, 6, 11, and 12. The numerical order of registers reflects the number of DNA residues of the primer substrate. Register 3 represents the RT-RNA/DNA complex in the absence of added dNTPs. The run-off product, generated in the presence of the four dNTPs, contains 27 DNA residues.

**RESULTS**

In order to analyze the interplay between RT polymerase and RNase H active sites on a PPT-derived RNA/DNA primer/template substrate, we first generated a series of stalled complexes, termed registers, through use of various dNTP/ddNTP combinations. Instead of employing a pure RNA primer that, because of the sequence 5′-CAGT-3′ immediately flanking the primer, leads to termination of DNA synthesis at positions +1, +2, +3, and +4, we devised a chimeric DNA-RNA primer (Pr + PPT 3D-17R) that yields chain termination at positions +4, +6, +11, and +12 (Fig. 1). This approach allowed us to study early events of the initiation reaction when RT is complexed with its initially bound RNA/DNA substrate as well as with chimeric replication intermediates. We also devised a DNA primer (Pr + PPT 20D) to generate a homologous DNA/DNA substrate for comparative purposes.

**Characterization of Stalled RT-RNA/DNA and RT-DNA/DNA Complexes**—The polymerization and RNase H cleavage products of differentially arrested RT-nucleic acid complexes are shown in Fig. 2. As expected, the 5′-end-labeled DNA primer was precisely elongated by 1, 3, 8, and 9 nt (left panel, lanes 4, 6, 11, and 12). The presence of all four dNTPs yielded a product of 44 nt in length (lane 27). The results obtained with the homologous RNA/DNA substrate are more complex (Fig. 2, right panel), since the RT polymerase and RNase H activities...
both use the same strand as substrate. Consistent with previous reports (16–18), RNase H cuts are seen at the DNA-RNA interface and further upstream, adjacent to the junction, at four consecutive positions, and between positions −7 and −8.

Precise extension with reasonable yield was observed only in register 4 when a single nt was added to the chimeric primer (Fig. 2, right panel, lane 4). This product was termed 4D-17R, with respect to the number of DNA and RNA residues of the extended primer. The expected reaction product in register 6, i.e. 6D-17R, is visible, but another shorter product, termed 1D-17R (see below), is also seen just above the RNase H cuts. Similar observations were made in registers 11 and 12 (lanes 11 and 12). In either case, the expected reaction products were less pronounced than the shorter ones, i.e. 2D-17R and 3D-17R.

**Primary and Secondary Initiation Reactions**—To characterize further the origin of the shorter reaction products, we next followed their formation in time course experiments. These were performed with dNTP/ddNTP combinations to yield registers 6 and 11 (Fig. 3A). The 6D-17R product appeared within the first few minutes and increased only slightly with longer reaction times (Fig. 3A, left panel). RNase H cuts at the junction (17R) and further upstream (16R and 15R) were seen clearly after 3 min, and the above-mentioned 1D-17R product, which migrated a little slower than the 17R cleavage band, first appeared between 12 and 20 min and further increased over longer incubation times. This result shows an order of product formation, i.e. first elongation to yield the 6D-17R product followed by RNase H cleavages and finally the formation of the 1D-17R product. RT thus initiates a second round of (+)-strand synthesis using the cleaved RNA fragment as a primer.

We will now use the term “primary reaction” and “secondary reaction” to distinguish between these two types of initiation events (schematically illustrated in Fig. 3C). During the primary reaction, incorporation of ddATP results in chain termination at position +6, whereas, during the secondary initiation reaction, ddATP is added at position +1 to yield a complex, termed register 1. Equivalent patterns were seen in registers 11 and 12 (Fig. 3A, right, and B, left); here, the stop-nucleotides (ddCTP and ddTTP) were added at positions +2 and +3 to yield the secondary 2D-17R and 3D-17R products.

The presence of dGTP should, in principal, allow elongation of RNA fragments that are generated by RNase H cleavages at the DNA-RNA junction and adjacent positions. Thus, the shorter secondary products may represent a heterogeneous mixture of chimeric strands, each of which contains a different number of DNA residues at the 3′-end. It can hardly be deduced from the above experiment which of the RNA fragments is preferentially used during the secondary initiation reaction. However, the observation that the 3D-17R product co-migrates exactly with the unextended primer indicates that the 17R cleavage product is most efficiently used (Fig. 3B, left panel). If shorter cleavage products would have been extended, the 3D-17R fragment would have migrated somewhat faster, due to the increased number of DNA residues in the chain-terminated product (see Fig. 2; the pure DNA oligonucleotide migrates faster than the chimeric DNA-RNA strand of the same length).

**Product Formation in the Presence of All Four dNTPs**—The above results showed that the initiation of (+)-strand synthesis is a complex process that involves primary and secondary initiation reactions, superimposed on the primer removal reaction. In order to understand better the natural reaction pathways, we next analyzed the order of product formation in the presence of all four dNTPs. The time course (Fig. 3B, right panel) shows that RT pauses at position +12, before DNA synthesis resumes to yield the run-off product (27D-17R). All reaction products, including the cleavage products (17R, 16R, and 15R), are already seen after the 1st min. The primer removal reaction appears to be equally efficient in the presence or absence of chain terminating nucleotides, as the enzyme encounters the template around position +12. For example compare Fig. 3A register 11, Fig. 3B register 12, and run-off synthesis. Thus, these data do not provide any information regarding the temporal relationship between the polymerase and RNase H active sites. The primer may be randomly cleaved at any point after initiation, immediately after synthesis of the run-off product, or alternatively the primer may already be removed once the 12th nt has been added. The above data do not enable us to distinguish among these various scenarios,
since cleaved 5'-end-labeled RNA fragments migrate at the same position in each case.

We therefore followed formation and processing of the initially synthesized product, using a 3'-end-labeled primer (Fig. 4). Putative secondary reactions are not detectable in this experiment, since the radiolabel is attached to the third DNA residue. The accumulation of the 12D-17R product, the pausing site, was again seen at early stages after initiation, i.e. 1 and 3 min. A relatively small fraction of this product is further extended to yield the unprocessed run-off product, which is later cleaved, as shown by the time-dependent decrease of this band. However, it seems that most of the 12D-17R reaction intermediate is prematurely cleaved to yield the 12D product. The 12D product then accumulates between 3 and 20 min and is later extended to yield the processed run-off product. Taken together, these data demonstrate that the primer is not randomly cleaved at any stage after initiation. The appearance of the single 12D product shows that the RNA primer is efficiently and precisely cleaved at the RNA-DNA junction after the 12th DNA residue has been incorporated.

Whether secondary initiation reactions also occur in the absence of chain-terminating stop-nucleotides cannot be answered on the basis of the above time course experiments. Synthesis of a secondary (+)-strand may be initiated after pausing and the following primer removal. Our data point to the existence of three different primer 3'-ends at position 112 that can potentially be recognized by the polymerase active site, i.e. the DNA 3'-end of the elongated unprocessed primer.

Fig. 3. A, time course of product formation of register 6 (left) and register 11 (right); B, time course of product formation of register 12 (left) and during synthesis of the run-off product (right). Reactions were monitored on the 5'-end-labeled chimeric primer. C, schematic representation of primary and secondary initiation events. Added nucleotides are underlined, and RNase H cuts are indicated by arrows. Registers that are attributable to secondary events are indicated with prime.

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**Retroviral (+)-Strand DNA Synthesis**

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(12D-17R), the DNA 3′-end of the elongated processed primer (12D), and the RNA 3′-end of the processed primer (17R). To determine which of these three 3′-ends might preferentially be used for DNA synthesis, we used a stoichiometric mixture of primer/template combinations with an uncleaved chimeric primer containing 17 RNA and 12 DNA residues and a nicked substrate with a 17-mer RNA primer and a 12-mer DNA primer. The former represents the substrate prior to primer removal, whereas the latter mimics the substrate after the RNase H cut at the RNA-DNA junction. The efficiency of DNA synthesis from each of the three available 3′-ends was compared in a competition experiment, using three separate reaction mixtures with 5′-end-labeled primers and an RNase H-deficient RT (Fig. 5A). The data show the following order of efficiency: 12D > 12D17R >> 17R. After a 60-min reaction, 55% of the 12D primer and 45% of the chimeric 12D-17R primer were extended to yield the final run-off product. In contrast, only 25% of the 17R was found to be extended, and most of the extended fraction accumulated after incorporation of the first nucleotide. These data show that secondary initiation reactions are clearly suppressed in the absence of a chain-terminated primary product and that RT preferentially elongates the 3′-end of the newly synthesized and processed DNA fragment. The additional pausing site at position 12. Moreover, synthesis of full-length (+)-strand DNA is still very efficient in this circumstance, indicating that the primer removal reaction is not required for polymerization steps after RT has reached position +12.

**Fig. 5.** A, efficiency of DNA synthesis of the various reaction intermediates at position +12, determined in a competition experiment. To mimic this stage of (+)-strand DNA synthesis, we used a stoichiometric mixture of a chimeric primer that represents the uncleaved extension product at position +12 (12D-17R) and a nicked substrate that contains the 17R and the 12D primer, in order to generate a substrate after the RNase H cut at the RNA-DNA junction. This mixture, the polymerase active site of RT could extend three different primer 3′-ends. Thus, the efficiency of DNA synthesis was compared using three separate reaction mixtures with 5′-end-labeled primers as tracers to follow the extension of the chimeric 12D-17R primer (left), the 17R RNA primer (center), and the 12D DNA primer (right). This is also schematically shown. The DNA template and the RNA primer are shown in black, and the DNA part of the chimeric primer and the 12D primer are shown in gray; the asterisk represents the radiolabel. Mixtures of preformed substrates (100 nM in each case) were incubated with the RNase H-deficient RT(E478Q) mutant enzyme (50 nM) to prevent any cleavages of the primer. Reaction times were 0, 1, 3, 6, 12, 20, 40, and 60 min. B, time course of DNA synthesis using the pure 5′-end-labeled 17R RNA primer. The preformed primer/template (100 nM) was incubated with the RNase H-deficient enzyme (50 nM) in the absence of the short 12D primer. The preformed complex with the 12D-17R primer was omitted from the reaction mixture to monitor primer extension in the absence of any competing substrates.

**Fig. 4.** Time course of DNA synthesis and RNase H cleavages using a 3′-end-labeled chimeric primer. Reactions were performed as described in Fig. 3. The lane on the left side shows 5′-end-labeled DNA oligonucleotides to assign cleaved fragments. Noteworthy, the 5′-end-labeled 12-mer DNA oligonucleotide and the pure DNA fragment generated through RNase H cleavage at the junction migrate exactly at the same position, since both of them contain 5′-PO₄ and 3′-OH groups.

**The Number of Base Pairs Accommodated between the Two Active Sites of HIV-1 RT with RNA/DNA and DNA/DNA Substrates**—We next analyzed the possible structural requirements for the specific pausing site at position +12. Pausing may be caused by the particular sequence or secondary structures of the single-stranded DNA template that inhibit the translocation of the enzyme. However, the specific pausing site after addition of the 12th nt is not observed with an homologous DNA/DNA substrate (Fig. 6), which indicates that pausing rather depends on the structure of the complexed chimeric
substrate and its interaction with RT. This conclusion is additionally supported by the observation that different template sequences, shown under experimental procedures, do not alter the pausing profile as long as DNA synthesis was initiated with the RNA primer (data not shown). To further approach this problem, we wished to define structural characteristics of complexes generated before, during, and after position 12. Studying RT-DNA/DNA complexes, we have recently shown that the interaction between the DNA template and the RT-associated RNase H can be monitored in the presence of Fe2⁺ and that Fe2⁺ binds to one of the metal-binding sites of the RNase H domain, thereby generating a high local concentration of hydroxyl radicals that might serve as active species to cause an oxidative strand break (15).

Here, we have used this tool to determine the number of base pairs between both active sites depending on the bound substrate, i.e. RNA/DNA or DNA/DNA. Although the purine-rich sequence of the dsDNA substrate used in this study differs markedly from the PBS-derived sequence used previously (15), the number of bp between the RT active sites was identical, i.e. 17 in each case (Fig. 7A, lane 1). In contrast, when RT was complexed with the homologous RNA/DNA substrate, a specific cut was seen on the DNA template at a distance of 18 bp upstream of the primer terminus (lane 2). Lower concentrations of cleaved products were seen when RT was bound to the RNA/DNA substrate, suggesting a diminished efficiency of cleavage, but the cleavage profile was exactly the same. However, the major product is shifted exactly by a single nucleotide when RNA/DNA is bound to RT. The small percentage of side products, i.e. less than 10%, can be explained by the oxidative cleavage mechanism that involves diffusible hydroxyl radicals as active species (15).

To provide additional information in regard to differences between RT-DNA/DNA and RT-RNA/DNA complexes, we also wished to study the protein-nucleic acid interface using hydroxyl radicals generated via Fe[EDTA]²⁻ as well as via ONOOK, as described previously (15, 19). However, a clear footprint could not be obtained, presumably reflecting the ability of RT to bind these substrates in both a polymerase-competent mode as well as one that facilitates removal of the primer (see “Discussion”). Despite the lack of a clear footprint, a faint band located on the DNA template strand, 7 bp upstream of the primer 3’-end, was seen when both complexes were treated with ONOOK (Fig. 7A, lanes 3 and 4). This cut was also seen as part of the ONOOK-dependent footprint on the DNA/DNA substrate derived from the PBS sequence (15). This particular reaction is thus a feature of polymerase-competent complexes and can be used as a second marker, in addition to the Fe²⁺ cut, to determine differences in the number of base pairs that fit in the substrate-binding channel. It is interesting to note that the

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**Fig. 6.** Time course of DNA synthesis using a 5'-end-labeled DNA primer. Reactions were performed as described in Fig. 3.

**Fig. 7.** A, location of Fe²⁺- and ONOOK cuts on the DNA template of RT-RNA/DNA and RT-DNA/DNA complexes. Cleavages were monitored on the 3'-end-labeled DNA template. Preformed complexes were incubated with ddGTP to form register 4. Chain-terminated complexes were then treated with Fe²⁺ and ONOOK as described under "Experimental Procedures." B, schematic representation of reactions in A. Lane C is a control incubation in the absence of RT and Fe²⁺ or ONOOK. Lane T is a T-ladder, generated with OsO₄-bipiridine. Lane C1 is the DNA/DNA substrate after incubation with Fe²⁺, and lane 1 represents the RT-DNA/DNA complex after the Fe²⁺ treatment. Lane 2 shows the Fe²⁺ cut on the DNA template of the RT-RNA/DNA complex. Lane C2 is the control reaction in the absence of RT. Lane C3 shows the DNA/ DNA substrate treated with ONOOK, and lane 3 shows the same reaction on the RT-DNA/DNA complex. Lane 4 represents ONOOK treatment of the RT-RNA/DNA complex, and lane C4 is the control, performed in the absence of RT. We note that a clear footprint was neither observed at longer exposures nor under reaction conditions that gave rise to stronger cleavages.
ONOOK cut appeared at the same position, regardless whether DNA/DNA or RNA/DNA was used as substrate. This observation indicates that the 1-bp variance is not homogeneously distributed over the entire duplex. Rather, it shows 7 bp between the primer 3′-end and the ONOOK cut, whereas a 1-bp difference (10 versus 11 bp) was seen upstream between the ONOOK and the Fe²⁺ cleavage sites (Fig. 7B).

Number of Base Pairs between Both Active Sites in Registers 4–17—The above data indicate that the number of base pairs located between the active sites of RTs might change when the enzyme has passed the initially bound RNA/DNA substrate and starts to accommodate more and more the newly synthesized DNA/DNA substrate. To address this issue, we next investigated registers 4, 6, 11, and 12 (Fig. 8, A and B) using the radiolabel now found at the 5′-end of the DNA template. In register 4, we observed the same cleavage positions on the DNA templates as seen in the previous experiment (see Fig. 7), revealing distances of 17 (Fig. 8A, lane 1) and 18 bp (lane 2), respectively, from the primer terminus. Thus, 3′- or 5′-end labeling does not influence the positions of the Fe²⁺ cuts, indicating an efficient and specific arrest of RT. Consistently, the DNA template of RT-DNA/DNA complexes in registers 6, 11, and 12 was always cleaved at a fixed distance of 17 bp from the primer terminus (lanes 3, 5, and 7), whereas the cleavage profiles on the corresponding RNA/DNA substrates are more complex. In register 6 (lane 4), the cut at position −12 was located 18 bp upstream of the 3′-end of the primer generated during the primary initiation event, whereas the cut at position −17 was located 18 bp upstream of the 3′-end of the primer generated during the secondary initiation event (see schematic in Fig. 8B). In registers 11 and 12, only a single cut was seen, corresponding to a distance of 18 bp upstream of the primer terminus of the secondary product (lanes 6 and 8). This is in full agreement with our analysis of product formation (Fig. 2) and confirms the existence of a secondary initiation complex.

To analyze whether the number of base pairs between both active sites will decrease to 17 nt at later stages after initiation, we next characterized stalled complexes in registers 12–17 (Fig. 9, A and B, schematically). These were generated using chimeric primers with 12, 14, and 16 DNA residues, respectively. The preformed complexes, containing 5′-end-labeled DNA templates, were incubated in the absence of Mg²⁺, to ensure that the RNA primer remains intact, which also excludes any secondary reactions. The next correct ddNTP was additionally added in the reaction mixture to stabilize the polymerase-competent complex. Under these reaction conditions, Fe²⁺ cuts were not observed in register 12, and only a very faint band, corresponding to a number of 17 bp, is seen in register 14. However, register 16 shows a clear cut at a distance of 17 nt from the primer terminus (lane 16), which corroborates the data obtained with pure DNA/DNA substrates. When these complexes were preincubated with Mg²⁺, which initiates the primer removal step and allows the addition of the single stop-nucleotide, the specific Fe²⁺ cuts on the DNA template clearly reappeared at distances of 18 and 17 nucleotides from the primer terminus (lanes 13 and 15).

Taken together, the presence or absence of a specific Fe²⁺ cut on the DNA template, as well as differences in its precise location, indicates structural differences between the various RT-nucleic acid complexes that are generated during the initiation of HIV (+)-strand synthesis. These data will be discussed in the context of our time course experiments that show the complex reaction pathway of initiation, pausing, primer removal, and finally continuation of DNA synthesis.

DISCUSSION

In this study we investigated the interplay between the polymerase and RNase H active sites of HIV-1 RT during the initiation of (+)-strand DNA synthesis. Based on time course experiments and biochemical studies focused on the characterization of the various RT-nucleic acid complexes involved in this process, i.e. RT bound to RNA/DNA and DNA/DNA primer/templates as well as complexes containing chimeric primers with 12, 14, and 16 DNA residues, the specific Fe²⁺ cuts on the DNA template that appear in accord with the extent of DNA synthesis identified the position of the RNase H active site when the polymerase active site lies in the vicinity of the primer 3′-end (Fig. 10). This result is consistent with biochemical data obtained with DNA/RNA, RNA/RNA, and DNA/DNA primer/template combinations (Refs. 8, 9, 11, 15, and, for recent review, Ref. 20) and demonstrates that RT binds all of its various nucleic acid substrates with the same orientation.

The RNase H domain remains positioned over the template strand, as RT starts to generate more and more DNA/DNA. Particularly, in the complex with 16 newly synthesized DNA residues, the specific Fe²⁺ cut on the DNA template is seen opposite the DNA-RNA junction of the primer strand. Thus, the RNase H active site as part of a polymerase-competent complex cannot interact simultaneously with the primer strand.

The time course of (+)-strand DNA synthesis shows that RT pauses after the 12th DNA residue has been added (step 2). At this point, the RT-associated RNase H activity starts to cleave the RNA primer from newly synthesized DNA (step 3), which, we propose, is accomplished by another RT molecule that is not involved in DNA synthesis.

This proposal is strongly supported by the RNase H cleavage profiles obtained with 5′- and 3′-end-labeled primer strands. A single cut at the DNA-RNA junction is only observed with the 3′-end-labeled primer, whereas the cleavage profile obtained with the 5′-end-labeled primer shows a time-dependent increase in shorter products. The shorter 5′-end-labeled fragments are thus identified as products that follow the initial cut at the junction. The primer removal reaction is thus accomplished in the 3′ → 5′ direction, whereas DNA synthesis proceeds in the opposite 5′ → 3′ direction. These data support the view that positioning of RT is reversed during the primer removal reaction (see step 3 and Refs. 22 and 23). In the latter studies, it has been suggested that RT binds close to the 5′-end of the RNA primer, such that the RNase H active site is positioned over the RNA-DNA junction. This is further supported by recent studies, showing that mutations in the “primer grip” region of HIV-1 RT selectively diminish this 5′-directed RNase H activity (24–26). Thus, the addition of 12 residues to the RNA primer may extend the growing strand sufficiently to facilitate binding of another competing RT molecule to the 5′-end of the primer that, in tum, initiates removal of the latter.

The observation that specific Fe²⁺ cuts on the DNA template are not seen in the complex with 12 DNA residues may provide additional support for the existence of an RNase H-competent
complex at this stage. However, we cannot exclude the possibility that the lack of Fe$^{2+}$ cuts may be attributable to altered RT-nucleic acid interactions in the polymerase-competent binding mode.

DNA synthesis finally resumes from the newly synthesized DNA fragment after the DNA-RNA junction, and adjacent positions are efficiently cleaved (steps 4 and 5). The time course experiments indicate that a fraction of the intact primer is also extended and later cleaved after synthesis of the run-off product (step 6). We further show that the enzyme is also capable of elongating the cleaved PPT primer, particularly when chain-terminating stop-nucleotides were incorporated in the primer 3'-end of both primary and secondary products and the Fe$^{2+}$ cut are indicated. Slashed arrows indicate the position of Fe$^{2+}$ cuts that should appear if the polymerase active site would interact with the 3'-end of the primary product instead of the primer terminus of the secondary product (see text).
ily synthesized DNA strand (step 7). We note that such secondary initiation reactions may critically limit the efficiency of nucleoside analog RT inhibitors, since reverse transcription could continue as long as cellular dNTPs are incorporated into the second strand.

A similar mechanism, designated “the (+)-strand primer recycling model,” has recently been suggested for yeast Ty retrotransposon reverse transcription (27). It has been proposed that synthesis of a secondary (+)-strand might displace the primary (+)-strand strong stop product, thereby facilitating removal of the tRNA primer. Accordingly, the secondary (+)-strand species does not contain any tRNA sequences but is subsequently used to facilitate the second strand transfer. This model is consistent with the observation that Ty elements do not inherit tRNA sequences, although the (+)-strand strong stop DNA that contains the tRNA copy has been identified as the most abundant product in Ty 1 reverse transcription (28, 29). We have now demonstrated secondary initiation reactions for the first time, using a cell-free assay. Our data support the general principal of a primer recycling model which may in fact be important for reverse transcription of retrotransposons.
shown that retroviruses inherit their tRNA sequences via the second strand transfer (30–32) which indicates that the primary synthesized (+)-strand product is involved in the second strand transfer. Therefore, a primer recycling mechanism may not be of relevance in regard to retroviral replication. This view is consistent with our in vitro data that show that secondary initiation reactions occur efficiently only when DNA synthesis of the primarily synthesized DNA strand is blocked after addition of a stop-nucleotide. In the presence of all four dNTPs, the polymerase active site preferentially extends the 3′-end of the newly synthesized DNA strand rather than the 3′-end of the cleaved RNA primer (Fig. 5).

Implications for a Conformational Change of the Bound Nucleic Acid—When RT initially binds the RNA/DNA substrate, oxidative Fe$^{2+}$ cuts were seen at a distance of 18 bp upstream from the primer terminus, whereas 17 bp were observed at later stages when the enzyme binds more and more DNA/DNA. This 1-base pair variance reflects structural differences between these complexes and further supports the idea that the number of base pairs that fit into the substrate binding groove between the two active sites of RT is an important criterion in determining differences in the conformation of complexed substrates (15).

Free in solution, RNA/DNA hybrids differ in local structural features from the classical A-form, but helical parameters that characterize the overall geometry of the helix, e.g. rise and twist, indicate an A-like conformation (33, 34). The pitch of an A-type helix is lower than that of a standard DNA/DNA B-form, and differences in twist angles between consecutive base pairs result in a 11-fold helix symmetry for the A-form, whereas a B-form helix contains 10 bp per turn (35). Therefore, a stretch representing the distance between the two enzymatic sites should accommodate about 3 more base pairs within a pure A-type helix than within a pure B-type helix. In accord with the crystal structure of the RT-dsDNA complex (12), we therefore suggest that the relatively small difference of 17 versus 18 bp accounts for the partial A-form of the complexed DNA/DNA duplex, whereas the RNA/DNA substrate is expected to retain the preferred A-type conformation.

Although we cannot exclude that subtle differences in the protein conformation or the kink of the bound dsDNA substrate may also affect our measurements, a further detailed comparison of our biochemical data in solution and the crystal structure of the RT-dsDNA complex show remarkable consistencies. According to the crystallographic data one would only expect differences in the number of base pairs in the upstream part of the complexed nucleic acids, since the DNA/DNA substrate adopts an A-conformation in the vicinity of the polymerase active site (12). This is supported by the finding that a specific cut on the DNA template, induced by ONOOK, is located exactly 7 bp upstream of the primer 3′-end, regardless whether DNA/DNA or RNA/DNA is used as a substrate. Thus, the 10 DNA/DNA bp versus the 11 RNA/DNA bp that are measured between the ONOOK and Fe$^{2+}$ cuts may represent precise differences of helical turns in B- versus A-forms (Fig. 11). These findings suggest that the initiation of (+)-strand DNA synthesis is accompanied by a conformational change of the bound nucleic acid substrate, i.e. the enzyme translocates from an initially bound A-type RNA/DNA substrate into the mixed A/B-conformation of the newly synthesized DNA/DNA.

Taken together, our data show that the PPT primer is cleaved immediately after addition of the 12th DNA residue and the ensuing pause event. Were the pausing of RT to be irrelevant in primer removal, and were the latter event to be determined only by the length of the growing strand, RNase H cleavage should be detected even after incorporation of the 12th nucleotide. However, DNA fragments consisting of 13 or more residues were not observed, and we instead detected a single specific band corresponding to the 12-mer product (Fig. 4). Thus, the RT pausing event at position +12 is likely one that facilitates primer removal at this stage of DNA synthesis. Additionally, as stated above, the 12 newly synthesized residues may represent the critical length of the growing chain that permits binding of the competing enzyme to the 5′-end of the primer. We further note that the pausing of the synthesizing RT involved in synthesis and the binding of the competing RT involved in primer removal are not necessarily independent events.

Finally, the time course data obtained with the RNase H-deficient enzyme show that the absence of the primer removal reaction does not prevent full-length synthesis of DNA. Regardless, the primer removal reaction is an important step to prevent reverse transcription of the PPT fragment at the very last steps of replication, when (+)-strand DNA synthesis resumes after the second strand transfer. Transcription of the primer at this stage would modify the retroviral U3 ends of the preintegrative DNA, which in turn would affect the entire integration process, since the activities of the viral integrase depend on the proper sequence and structure of both ends of the dsDNA (36). Therefore, the early removal of the (+)-strand primer, after addition of the 12th DNA residue, might be of importance to ensure correct reverse transcription and integration.

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