Structures of Complexes Formed by HIV-1 Reverse Transcriptase at a Termination Site of DNA Synthesis

Marc Lavigne‡, Lucette Polomack§, and Henri Buc¶
From the Unité de Physicochimie des Macromoléculles Biologiques, Institut Pasteur, CNRS URA 1773, 75724 Paris Cedex 15, France

This study presents structural parameters associated with termination of human immunodeficiency virus, type 1 (HIV-1) reverse transcriptase (RT) at Ter2, the major termination site located in the center of the HIV-1 genome. DNA footprinting studies of various elongation complexes formed by RT around wild type and mutant Ter2 sites have revealed two major structural transformations of these complexes when the enzyme gets closer to Ter2. First, the interactions between RT and the DNA duplex are less extended, although the global affinity of the enzyme for this duplex is only decreased by 2-fold. Second, there is an atypical positioning of the RT RNase H domain on the DNA duplex. We interpret our data as indicating that the $A_nT_m$ motif located upstream of Ter2 prevents a classical positioning of the enzyme on the double-stranded part of the DNA duplex at some precise positions of elongation downstream of this motif. Instead, novel species of binary and/or ternary complexes, characterized by atypical footprints, are formed. The new rate-limiting step of the reaction, characterized in the preceding paper (Lavigne, M., Polomack, L., and Buc, H. (2001) J. Biol. Chem. 276, 31429–31438), would be a transition leading from these new species to a catalytically competent ternary complex.

DNA polymerases have a common chemistry of nucleotide incorporation, and the structures involved in the polymerization process are mostly conserved. This conservation, initially suggested by the identification of conserved motifs (1), has been confirmed by numerous structural and biochemical studies performed on these enzymes (reviewed in Refs. 2–5). As originally described for the large fragment of Escherichia coli DNA polymerase I (Klenow fragment) (6), the general shape of these polymerases resembles a right hand with “fingers,” “thumb,” and “palm” domains. While the palm domain contains the residues responsible for the catalysis of polymerization, the other domains are also involved in this process, for example by interacting with the nucleic acid or with the incoming nucleotide. However, DNA polymerases display some structural differences. Although the “right hand” shape is conserved, there are some differences in the underlying primary sequence, in the secondary structure, and in the size and oligomeric state. Also, the parameters associated with the polymerization process (such as the fidelity, the overall rate, the processivity, and the sensitivity to various drugs) are different for each polymerase. As a consequence, DNA polymerases are expected to display subtle variations around a common theme.

Among DNA polymerases, HIV-1 reverse transcriptase (RT) is remarkable for the variety of functions it assumes during the copy of the retroviral genome (for recent reviews, see Refs. 7–9). It catalyzes DNA polymerization on both DNA and RNA templates and degrades the RNA template using its RNase H activity. It can also initiate DNA synthesis from a specific DNA (10–14) or from small oligoribonucleotides (called poly-A or poly-T) (15–17) and has the ability to “jump” during DNA synthesis from one RNA template to another (18–26). Finally, HIV-1 RT terminates the synthesis of the DNA (+)-strand at two precise sites (Ter1 and Ter2) located in the center of HIV-1 genome. Termination at these sites is important for HIV-1 infectivity, and the central termination sequence acts as a cis-determinant of HIV-1 DNA nuclear import (27, 28). $A_nT_m$ motifs are located at the 3′-end of the termination sites, and the narrowing of the DNA minor groove induced by these motifs is responsible for the termination events (29, 30).

Most of the kinetic studies on HIV-1 RT have been performed during classic elongation processes on both RNA and DNA templates. These studies have shown that the mechanism of elongation by HIV-1 RT is similar to the one proposed for other DNA polymerases. In the simplest case, this mechanism is sequential and requires a conformational change of the enzyme, which precedes the chemical reaction and corresponds generally to the rate-limiting step of the reaction (31–34) (see Scheme I of the preceding article (60)). On the basis of crystal structures of HIV-1 RT (for example, see Refs. 35–37) and in particular the recently solved ternary complex containing the enzyme, a covalently attached DNA duplex, and dTTP (38), a three-dimensional interpretation of this sequential mechanism has been formulated (7). This model does not conflict with the types of movements that have been suggested for the faithful incorporation of a substrate by the polymerase $\beta$- or the polymerase I-like families of enzymes (see, for example, the structures of T7 DNA polymerase (39) and KlenTaq (40) and the reviews (2–4)). This interpretation proposes that the initial binding of RT to the nucleic acid is associated with a large rotation of the “thumb” domain away from the “fingers” domain of $p66$. The slow conformational change of RT that precedes the chemical reaction would correspond to a closing movement of the “fingers” domain around the incoming nucleotide. Structural equivalents of the initial binary complex $E-D_n$-
Footprinting studies have completed this structural information. The "canonical" footprint of a binary complex E-D₃, formed at a position of processive synthesis is characterized by the DNA areas protected against cleavage by DNase I (+7 to −23 on the template strand and −1 to −25 on the primer strand) or by hydroxyl radicals (+3 to −15 on the template strand and +1 to −15 on the primer strand) and by sites hypersensitive to cleavage by these nuclease (positions −20 and −17 on the template strand, respectively) (41, 42). Hydroxyl radicals footprints have also revealed a useful property of HIV-1 RT; the Fe²⁺ cation used to generate the hydroxyl radicals can replace the catalytically active Mg²⁺ of the RNase H domain of RT. These radicals cleave the most proximal base of the DNA (43). The Fe²⁺-dependent cuts have been used to trace the activity of the RNase H domain on various DNA/RNA hybrids and DNA/DNA duplexes that reproduce different stages of the replication cycle (15). Finally, the use of mutated RTs that can be site-specifically modified with a photoactivable cross-linking agent or cross-linked with modified nucleotides has allowed the characterization of specific contacts between side chain residues of RT and the nucleic acid duplex (e.g. see Refs. 44 and 45).

It is important to know how the structures of the complexes formed by the enzyme during processive synthesis are affected at the steps where the mechanism of elongation is strongly perturbed. Footprinting and kinetic studies performed on the initiation steps have already explained the specificity of these processes (13, 15). In the case of termination at the central termination sequence, the kinetic studies presented in the preceding article (60) have shown at least three properties of the relevant complexes. First, at the termination site Ter2 or its immediate vicinity, the global affinity of HIV-1 RT for the corresponding duplex is still in the nanomolar range. Second, a change in the mechanism occurs in a very narrow interval of three nucleotides (at Ter2 and two positions downstream). Third, termination at Ter2 results from a large decrease in the rate of a step located after the formation of an initial ternary complex (13, 15). In the case of termination at the central position of processive synthesis is characterized by the ternary intermediate (E-D₃−dNTP in the classical formalism) and before the dissociation of the enzyme from the elongated duplex. This step can be the chemical step and/or the usual conformational isomerization prior to this step. Conversely, termination could also result from a more profound perturbation of the classical mechanism of nucleotide incorporation.

In this report, footprinting methods were used to determine the structure of complexes formed by RT at Ter2 and in its vicinity. These studies, performed on small DNA duplexes, have revealed a large heterogeneity in the complexes formed during polymerization at this termination site. They have also shown how a narrow minor groove positioned upstream of the polymerization site could affect the positioning of RT on the corresponding duplex. Indeed, we have found that the most stable complex formed by RT at Ter2 is characterized by an atypical positioning of the RNase H domain on the synthesized DNA.

EXPERIMENTAL PROCEDURES

Oligonucleotides, Proteins, and Buffers—Oligodeoxyribonucleotides (called oligonucleotides) were purchased from Genset and purified by preparative electrophoresis to more than 95% homogeneity. dNTP and ddNTP were from Amersham Pharmacia Biotech.

HIV-1 RT was generously given by T. Unge (46). Its active site titration was determined, according to Ref. 31, on an extended PG5/D22 duplex.

Duplexes used in kinetic and footprinting assays were prepared following the same protocol. One of the two oligonucleotides (primer or template) is labeled at its 5’-end with 32P. The two oligonucleotides are annealed in a 2:1 ratio of cold to labeled oligonucleotides. Annealing consists of an incubation of the duplex at 75°C for 5 min, followed by a slow cooling, in a 100 mM Tris-HCl (pH 7.8), 400 mM NaCl, 8% (v/v) polyethylene glycol 6000 solution. Concentrations of the duplexes were always at least 20 times higher than their concentrations in the final assay.

Footprinting assays—Three different probes were used in these footprinting assays. DNase I attacks were performed as described (47) at final concentrations of 2 and 2.5 μg/ml for duplexes labeled in their primer or template strands, respectively. Hydroxyl radical attacks generated by the Fe–EDTA complex and Fe²⁺–complexed with the RNase H domain were performed for 1 min at 37°C, following a procedure described in Refs. 41 and 43, respectively. In the presence of RT, final concentrations of iron, EDTA, ascorbate, and H₂O₂ were 1 mM, 1 mM, 1 mM, and 0.05% respectively. In the absence of RT, these reagents were used at half of these concentrations. Cleavage products, after precipitation with ethanol, were analyzed on 12 or 16% polyacrylamide sequencing gels. When required (titration experiments), the cleavage products were quantified using PhosphorImaging and the ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Oligonucleotides and Strategy of Structural Analysis—In order to characterize the structure of the complexes formed by HIV-1 RT at different positions around WT and mutant Ter2 sites, various DNA/DNA duplexes were constructed (Fig. 1). One strand of each duplex (template or primer) was labeled at its 5’-end. Three different probes were used to obtain the footprints of the complexes formed between RT and DNA duplexes at equilibrium: DNase I, hydroxyl radicals generated by the Fe–EDTA complex, and hydroxyl radicals generated by Fe²⁺ located within the RNase H catalytic site of the enzyme (called Fe²⁺–RNase H complex). On each duplex, RT-DNA complexes were formed under two different conditions. The first one consists of a mixture of RT, DNA duplex, and an incorporable ddNTP. This condition allows formation of the binary complex E-D₃. Under the second condition, a dNTP complementary to the next position is added but cannot be incorporated because there is no OH residue at the 3’-end of the primer strand.
In most cases, this last condition allows formation of a ternary complex, which is more stable than the binary complex (31, 48 and previous article) and which is thought to mimic the active enzyme ternary complex (E-D_n-dNTP) frozen just prior to phosphodiester bond formation. The unincorporated dNTP is also called a stabilizing nucleotide. However, this situation is not general, and the ternary complexes prepared under these conditions can be assigned to other types of E-D_n-dNTP complexes, as we show later.

FIG. 2. DNase I and hydroxyl radical footprints of complexes made by HIV-1 RT at various positions around the WT Ter2 site. Duplexes made with a WT 60-mer oligonucleotide as template strand and various complementary oligonucleotides as primer strands (described in Fig. 1) were elongated at 37 °C by HIV-1 RT (30 nM enzyme for 5 nM duplex) and various combinations of dNTP and ddNTP (250 μM). These conditions allow formation of RT-DNA complexes at different positions of elongation around the WT Ter2 site. More precisely, complexes at positions 4930 and 4931 are obtained by elongation of the duplex f4929/f60 with ddATP and dATP respectively; complexes at positions 4932, 4933, and 4934 are obtained by elongation with ddTTP of duplexes f4931/f60, f4932/f60, and f4933/f60, respectively; and complexes at positions 4935 and 4936 are obtained by elongation of duplexes f4933/f60 and f4935/f60 with dTTP and ddCTP respectively. The times of elongation were adjusted to obtain an optimal percentage of RT-DNA complex at the desired position. At each position, the effect of the addition of the next incorporable dNTP has been examined. In this case, 250 μM of dNTP was added for 5 min. This stabilizing dNTP is dTTP at positions 4930–4933, dCTP at position 4934, and dGTP at positions 4935 and 4936. The RT-DNA complexes formed in the absence and presence of stabilizing nucleotide correspond to binary E-D_n and ternary E-D_n-dNTP complexes. Footprints presented in A–C were performed on duplexes where the template strand is 5'-32P-labeled. Footprints presented in Fig. 2D were obtained on duplexes where the primer strand is 5'-32P-labeled. RT-DNA complexes and free DNA were attacked by three different probes (details under “Experimental Procedures”). These probes are DNase I (A and D) and hydroxyl radicals generated by the Fe-EDTA complex (B) or by the Fe-RNase H complex (C). In A and D, the regions of the duplex protected against DNase I are indicated by dashed lines, and the DNase I hyperreactive sites are indicated by arrows.
Footprints of Complexes Formed by HIV-1 RT at Various Positions around the WT Ter2 Site in the Absence and Presence of Stabilizing Nucleotide—Fig. 2, A–D, presents the footprints of the complexes formed at different positions around WT Ter2, obtained using DNase I (Fig. 2, A and D), hydroxyl radicals generated by the Fe-EDTA complex (Fig. 2B), or hydroxyl radicals generated by the Fe-RNase H complex (Fig. 2C). Fig. 3 gives a synopsis of these footprints. Positions of elongation are always designated by the location of the primer 3′-end on the HIV-1 genome. Positions of cleavage by DNase I or by hydroxyl radicals are numbered in relation to the location of this 3′-end. For example, when elongation terminates at position 4934 (Ter2), the nucleotide attacked by hydroxyl radicals and located at position 4934 is numbered −1, and the phosphodiester bond cleaved by DNase I and located between nucleotides 4933 and 4934 is numbered −1.5.

The binary complexes formed around WT Ter2 in the absence of stabilizing nucleotide are characterized by the heterogeneity of their footprints.

At positions 4931 and 4936, three nucleotides upstream and two nucleotides downstream of Ter2, these footprints are very similar to the “canonical” footprints of a binary complex formed at positions where elongation is processive (41–43). They show a large protection from DNase I cleavage, which extends to position −25.5 on both template and primer strands and a hyperreactivity at positions −21.5 and −18.5 on template and primer strands, respectively. Footprints with hydroxyl radicals reveal a hyperreactivity at position −17 on the template strand whatever the origin of the radicals (Fe-EDTA or Fe-RNase H complex). Footprints with hydroxyl radicals generated by the Fe-EDTA complex also show a protection of the template strand around the elongation site. Quantification of the hydroxyl radical cleavage products indicates an important decrease of cleavage between positions −6 and +1 (data not shown).

At positions 4932 and 4933, two and one nucleotides upstream of Ter2, some characteristics of the “canonical” footprints are changed especially when hydroxyl radicals generated by the Fe-EDTA complex are used as a probe (loss of hyperreactivity at position −17 or decrease of protection around the elongation site). However, the characteristics of the DNase I footprints and the hyperreactivity toward hydroxyl radicals generated by the Fe-RNase H complex are maintained.

Therefore, the binary complexes formed at the two positions located immediately upstream of Ter2 have footprints very similar to the footprints of a canonical E-Dn complex.

Finally, the footprints of the binary complexes formed at positions 4930, 4934, and 4935 display many differences with the previous set of data. At position 4930, located exactly between Ter1 and Ter2, the footprint is characterized by a smaller protection against DNase I cleavage and by the loss of hyperreactivity to DNase I and hydroxyl radicals at the canonical positions. Hyperreactivity to hydroxyl radicals generated by the Fe-RNase H complex is observed on the template strand at positions −14 and −11 instead of position −17, suggesting an atypical positioning of the RNase H domain of the enzyme on the DNA/DNA duplex. At positions 4934 and 4935, which correspond to Ter2 and one nucleotide downstream, footprints of binary complexes are characterized by a weak and short protection against DNase I cleavage and by a hyperreactivity of nucleotides −13 to −11 on the template strand to hydroxyl radicals generated by the Fe-RNase H complex.

A footprinting study of the ternary complexes, formed in the presence of stabilizing nucleotide, has also been performed at various positions around Ter2. At positions 4930–4933 and at position 4936, the footprints are very similar to those of canonical elongation complexes. We attribute them to the E-Dn-dNTP complexes, which precede the chemical reaction when elongation is performed at a processive position. On the other hand, footprints of complexes formed at positions 4934 and 4935 are different. Protection against DNase I cleavage is more restricted and is associated with a loss of hyperreactive sites on both strands of the duplex. The classical protections and hyperreactivities due to hydroxyl radicals are also lost. They are replaced by a hyperreactivity of the nucleotides located on the template strand between positions −14 and −11.

The negative effect exerted by the A,T tract on the structure of the binary and ternary complexes formed around Ter2 can then be summarized as follows.

First, this effect is very local. If one excludes the footprints of the binary complex formed at position 4930, which can be explained by the combined effects of Ter1 and Ter2, the footprints of all complexes are largely different from canonical footprints at two positions only: Ter2 and one nucleotide downstream. This window is one nucleotide smaller than the window where a decrease of the k_diss was observed in the recycling...
Termination of DNA Synthesis by HIV-1 RT: Footprinting Studies

Fig. 4. DNase I and hydroxyl radical footprints of complexes formed by HIV-1 RT at various positions around the m-C2 Ter2 site (template strand). A duplex made with a 5'-32P-labeled 60-mC2 template and the complementary f4933 primer was elongated at 37 °C by HIV-1 RT (30 nM enzyme for 5 nM duplex) and various combinations of dNTP and ddNTP (250 μM). RT-DNA complexes are formed at positions 4934, 4935, and 4936 by elongation of the duplex with dTTP, dCTP, and dGTP, respectively. Protocols similar to the ones given in the legend to Fig. 2 were followed. A complex formed at position 4933 between RT, the duplex, and no nucleotide was also analyzed (lanes 3 and 3'). These RT-duplex complexes and the free duplex were attacked by two different probes: DNase I (left panel) and hydroxyl radicals generated by the Fe-RNase H complex (right panel). The regions of the template strand protected against DNase I are indicated by dashed lines, and the phosphodiester bonds hyperreactive to DNase I are indicated by arrows.

The binary complexes formed at these positions show three major differences with canonical binary complexes: a weaker interaction between RT and the DNA duplex, the loss of some contacts between the RNase H domain of the enzyme and the upstream part of the duplex, and the existence of new contacts between this domain and another part of this duplex. These characteristics define a new type of binary complex, called here an incomplete binary complex. At Ter2, this complex is also sensitive to heparin (see preceding article (60)).

Second, the negative effect exerted by the A₉T₇ motif on the formation of the binary complexes is partially reversed in the presence of a stabilizing nucleotide. At positions 4932 and 4933, this reversion is total and leads to a complex having canonical footprinting characteristics (hyperreactivity to hydroxyl radicals generated by the Fe-EDTA complex) observed at position −17 on the template strand. At positions 4934 and 4935, the tertiary complexes show a stronger protection against DNase I cleavage. However, they do not yield an extended footprint, and they are still characterized by an atypical positioning of the RNase H domain on the DNA duplex.

The binary complexes formed at these positions have a stronger protection against DNase I cleavage. This structural observation is limited to the probes used in this study.

Therefore, mutations m-C2 and m-C12, which widen the DNA minor groove of the A₉T₇ motif and abolish termination at Ter2, also restore the formation of ternary complexes having a classical structure at positions 4934 and 4935. However, at these two positions, the mutations do not allow a full recovery of the structure of the canonical binary complexes (as observed at position 4933). They are still characterized by an atypical positioning of the RNase H domain of the enzyme on the DNA duplex. This structural information is consistent with the kinetic studies performed on analogous duplexes, which have shown that mutations m-C2 and m-C12 do not restore full processivity at Ter2.

Focuses and affinity and positioning of the RNase H Domain Characterizing the Ternary Complexes Formed on WT and m-C2 Duplexes—

The major structural differences observed between the ternary complexes formed at WT Ter2 and at more processive positions can then be used to quantify the amounts of each of these complexes formed at different concentrations of RT. This study was performed on three different duplexes: f4929/f60 WT and f4933/f60 m-C2, which allow formation of a ternary complex at a posi-

generated by the Fe-RNase H complex. The footprints observed on the two mutated duplexes are very similar, so we only present the ones observed on the m-C2 duplex. (Fig. 4).

The binary complexes formed at positions 4934–4936, in the absence of stabilizing nucleotide, show a complete and canonical protection of the DNA against DNase I cleavage. However, at positions 4934 and 4935, the canonical hyperreactive cleavages generated by DNase I and hydroxyl radicals are not observed. Hyperreactive cleavages by hydroxyl radicals are observed instead at positions −18 and −15 when the complex is formed at Ter2 and at positions −16, −15, −7, and −6 when the complex is formed one nucleotide downstream. On these mutant duplexes, the ternary complexes formed at positions 4934–4936 display all of the footprinting characteristics of canonical elongation complexes. This structural observation is limited to the probes used in this study.

Therefore, mutations m-C2 and m-C12, which widen the DNA minor groove of the A₉T₇ motif and abolish termination at Ter2, also restore the formation of ternary complexes having a canonical structure at positions 4934 and 4935. However, at these two positions, the mutations do not allow a full recovery of the structure of the canonical binary complexes (as observed at position 4933). They are still characterized by an atypical positioning of the RNase H domain of the enzyme on the DNA duplex. This structural information is consistent with the kinetic studies performed on analogous duplexes, which have shown that mutations m-C2 and m-C12 do not restore full processivity at Ter2.

Affinity and positioning of the RNase H Domain Characterizing the Ternary Complexes Formed on WT and m-C2 Duplexes—

The major structural differences observed between the ternary complexes formed at WT Ter2 and at more processive positions can then be used to quantify the amounts of each of these complexes formed at different concentrations of RT. This study was performed on three different duplexes: f4929/f60 WT and f4933/f60 m-C2, which allow formation of a ternary complex at a posi-

elongation assays presented in the accompanying article. The binary complexes formed at these positions show three major differences with canonical binary complexes: a weaker interaction between RT and the DNA duplex, the loss of some contacts between the RNase H domain of the enzyme and the upstream part of the duplex, and the existence of new contacts between this domain and another part of this duplex. These characteristics define a new type of binary complex, called here an incomplete binary complex. At Ter2, this complex is also sensitive to heparin (see preceding article (60)).

Second, the negative effect exerted by the A₉T₇ motif on the formation of the binary complexes is partially reversed in the presence of a stabilizing nucleotide. At positions 4932 and 4933, this reversion is total and leads to a complex having canonical footprinting characteristics (hyperreactivity to hydroxyl radicals generated by the Fe-EDTA complex) observed at position −17 on the template strand. At positions 4934 and 4935, the ternary complexes show a stronger protection against DNase I cleavage. However, they do not yield an extended footprint, and they are still characterized by an atypical positioning of the RNase H domain on the DNA duplex.

The binary complexes formed at these positions have a stronger protection against DNase I cleavage. This structural observation is limited to the probes used in this study.

Therefore, mutations m-C2 and m-C12, which widen the DNA minor groove of the A₉T₇ motif and abolish termination at Ter2, also restore the formation of ternary complexes having a canonical structure at positions 4934 and 4935. However, at these two positions, the mutations do not allow a full recovery of the structure of the canonical binary complexes (as observed at position 4933). They are still characterized by an atypical positioning of the RNase H domain of the enzyme on the DNA duplex. This structural information is consistent with the kinetic studies performed on analogous duplexes, which have shown that mutations m-C2 and m-C12 do not restore full processivity at Ter2.

Affinity and positioning of the RNase H Domain Characterizing the Ternary Complexes Formed on WT and m-C2 Duplexes—

The major structural differences observed between the ternary complexes formed at WT Ter2 and at more processive positions can then be used to quantify the amounts of each of these complexes formed at different concentrations of RT. This study was performed on three different duplexes: f4929/f60 WT and f4933/f60 m-C2, which allow formation of a ternary complex at a posi-
FIG. 5. DNase I and hydroxyl radical footprints of RT-DNA complexes formed on WT and m-C2 duplexes at various concentrations of RT. Three DNA duplexes (f4929-WT/f60-WT, f4933-WT/f60-WT, and f4933-mC2/f60-mC2) made with a 5'/H11032-32P-labeled template strand, were elongated at 37 °C for 15 min by HIV-1 RT using ddATP + dTTP (first duplex) or ddTTP + dCTP (two last duplexes). These conditions generate ternary complexes at positions 4930 and 4934 on the WT sequence and at position 4934 on the m-C2 sequence. These ternary complexes were formed at one concentration of duplex (1 nM), at various concentrations of enzyme (0 nM, lanes 3a, 3b, 3c, and 4c; 0.2 nM, lanes 5a, 5b, and 5c; 0.4 nM, lanes 6a, 6b, and 6c; 0.8 nM, lanes 7a, 7b, and 7c; 1.6 nM, lanes 8a, 8b, and 8c; 3.2 nM, lanes 9a, 9b, and 9c; 6.4 nM, lanes 10a, 10b, and 10c; 12.8 nM, lanes 11a, 11b, and 11c; 25.6 nM, lanes 12a, 12b, and 12c) and at the same concentration of nucleotides (250 μM). These complexes were attacked by DNase I (Fig. 5A) or by hydroxyl radicals generated by the Fe-RNase H domain (Fig. 5B), the cleavage products being then separated on an 8% polyacrylamide sequencing gel. DNase I cleavage products were quantified at positions 4923.5–4925.5 (1), 4926.5–4928.5 (1'), 4929.5–4931.5 (1''), 4915.5–4919.5 (2), 4906.5 (3), and 4904.5 (3') (Fig. 5A). Hydroxyl radical cleavage products were quantified at positions −17 and −13 to −11, with the numbering referring to the 3'-end of the primer (Fig. 5B). The percentages of cleavage measured at these positions were normalized and plotted versus the concentration of RT (Fig. 5C).
tion where synthesis is processive, and f4933/60 WT, which allows formation of a ternary complex at Ter2. The corresponding footprints obtained with DNase I and hydroxyl radicals generated by the Fe-RNase H complex are presented in Fig. 5, A and B.

At the two first positions (4930 on the WT sequence and 4934 on the m-C2 sequence), a large and clear protection against DNase I is observed at 0.8 nM RT (lanes 8a and 8c). This protection extends up to positions −24.5 and −21.5, respectively, and is associated with a hyperreactivity of hydroxyl radicals at position −17. This hyperreactivity also appears after the addition of 0.4–0.8 nM RT. At the WT Ter2 site, the protection against DNase I requires a slightly higher concentration of RT (lanes 9b and 10b). This protection is weaker in the upstream part of the footprint and is not associated with a hyperreactivity to hydroxyl radicals at position −17 (very faint band). Instead, a reactivity to hydroxyl radicals is observed between positions −13 and −11. Therefore, the ternary complexes formed in the presence of a stabilizing nucleotide at WT Ter2 and at positions where synthesis is more processive have similar affinities (in the nanomolar range) but different structures. As already observed at a single concentration of enzyme (Figs. 3 and 4) and now emphasized at the highest concentrations in this titration, these differences correspond to the loss of specific and tight protection by RT of the upstream part of the duplex and to an atypical positioning of the RNase H domain.

In order to evaluate more precisely these differences, products of cleavage by DNase I and hydroxyl radicals were quantified from the gels presented in Figs. 5, A and B (Fig. 5C). Percentages of DNase I cleavage were calculated in three different regions of the duplexes on the template strand. Regions 1′, 1′, or 1′ and 2 are located about one and two DNA helix turns from the 3′-end of the primer, respectively. The percentages of DNase I cleavage in these regions were normalized with respect to the percentage of DNase I cleavage calculated in a third region, where the percentage of cleavage does not change with the concentration of RT (region 3 for the complex formed at position 4930 on the WT sequence and region 3′ for the complexes formed at position 4934 on the WT or m-C2 sequence). Similarly, the percentages of hydroxyl radical cleavage were calculated at two positions of the duplexes (−17 and −13 to −11), and the ratio between these cleavage percentages is plotted at the different concentrations of RT. This approach compensates for the fluctuations that are sometimes observed between the percentages of hydroxyl radical cleavage (see for example, lane 8a of Fig. 5B).

As expected, differences between the ternary complex formed at WT Ter2 and the complexes formed at the two processive positions are clearly manifested in this analysis (Fig. 5C). At WT Ter2 (position 4934), the percentages of DNase I cleavage calculated in regions 1′ and 2 show a sigmoidal decrease when the concentration of enzyme increases. Inflection points are observed at a similar concentration of RT (−2 nM), but the plateau values at high concentrations of RT are different (full and 60% protections of region 1′ and 2, respectively). The percentages of hydroxyl radical cleavage also show a decrease by 2-fold when the concentration of RT is increased, with a midpoint also located around 2 nM. At the two other positions (4930 on the WT sequence or 4934 on the m-C2 sequence), the percentages of DNase I cleavage in regions 1, 1′, and 2 also decrease as the concentration of RT increases. However, the inflection point is located at a lower concentration of RT (−1 nM). Maximal protection is observed on both duplexes at high concentrations of RT. Finally, the percentage of hydroxyl radical cleavage shows a 10-fold increase with increasing RT concentrations, and the midpoint is located around 1–2 nM.

These values strengthen the qualitative conclusions presented above. First, at WT and mutant Ter2 sites, the constants that characterize the dissociation of the enzyme from the ternary complex at 250 μM dNTP and measured by footprinting techniques are very close to the equilibrium dissociation constants of the binary complexes measured by a kinetic method (K_{D, E} (cf. Table II in the preceding article (60)). Second, the mutation m-C2 has a small effect on the values of K_{D, E} measured at Ter2 by both the kinetic and footprinting approaches. These results confirm that the negative effect exerted by the A_{T, m} motif on elongation is not the result of lower affinities of RT for the DNA duplex in the binary and ternary complexes formed at Ter2. At this position, the enzyme forms tight and specific complexes that preclude the establishment of classical elongation complexes. However, we cannot exclude that a low percentage of these classical intermediates are present and that they escape detection by the footprinting methods used in this study.

Differential Effect of dCTP on the Formation of Ternary Complexes by HIV-1 RT at the WT and m-C2 Ter2 Sites—The structural differences identified between the ternary complexes formed at the WT and m-C2 Ter2 sites have also been analyzed at different concentrations of stabilizing nucleotide. Using the same probes as above, we compared the extent of complex formation on the f4933/60 WT and m-C2 duplexes, in the presence of ddTTP (250 μM) and dCTP (concentrations ranging from 1 to 1000 μM). These footprints are presented in Fig. 6, A and B.

The DNase I footprints (Fig. 6A) show that the weak protection of the upstream part of the WT duplex (region 2), observed in the absence or at a low concentration of dCTP, becomes stronger at higher concentrations of dCTP (1 mM). It becomes nearly similar to the protection observed on the mutant duplex. This effect is more striking when the intensity of these cleavages is normalized as above.

The effects of increasing dCTP concentration on the extent of cleavage due to hydroxyl radicals generated by the Fe-RNase H complex are different on the WT and m-C2 duplexes (Fig. 6B). On the WT duplex, a cleavage is observed around positions −11 to −13 in the presence of low concentrations of dCTP (maximum at 30 μM). This cleavage disappears and is replaced by a faint cleavage at position −17 at high concentrations of dCTP (1 mM). On the m-C2 duplex, in the absence or at low concentration of dCTP (1 μM), faint cleavages are observed at positions −18 and −15. For dCTP concentrations above 10 μM, these cleavage sites are rapidly replaced by a strong and unique cleavage at position −17.

These results show that the atypical footprints of the ternary complex formed at WT Ter2 disappear when the concentration of stabilizing nucleotide is increased. A strong protection of the upstream part of the WT Ter2 duplex against DNase I and a conventional positioning of the RNase H domain on this duplex are restored at 1 mM dCTP. This value is significantly larger than the dissociation constant K_{D, RNase H} of dCTP from the ternary complex present before the rate-limiting step and measured by kinetic studies (150 μM; cf. the preceding article (60)). This difference suggests that, at low concentrations of dCTP, elongation can proceed at Ter2, albeit slowly, without the establishment of canonical footprints.

DISCUSSION

The kinetic studies presented in the preceding article (60) have shown that termination of DNA synthesis at Ter2 is mainly due to a large decrease in the rate of a step located after the formation of the ternary complex at Ter2 and before the dissociation of the enzyme at Ter2 +1. In order to understand this effect at a structural level, footprints of different complexes
formed at equilibrium at Ter2 were analyzed and compared with the footprints of homologous complexes formed at positions where synthesis is more processive. Combinations of results obtained with kinetic and footprinting methods are generally complementary. However, the kinetic studies concern only the RT-DNA complexes engaged in elongation process and can give a simplified overview of this process. On the other hand, footprinting studies performed at equilibrium examine all of the complexes. Among this population, the structure of some intermediates could remain undetected, and these studies may favor the observation of intermediates that are not engaged into the polymerization process. Assuming these restrictions, we have combined the results obtained with both kinetic and footprinting methods and proposed a mechanism of termination of DNA synthesis at Ter2.

**New Species of RT-DNA Complexes Are Formed at Ter2 and One Nucleotide Downstream of Ter2, Where Elongation Is Highly Perturbed**—At Ter2 and Ter2 +1, where the enzyme faces its more difficult task, the footprints of the binary and ternary complexes are extremely informative. Both complexes are characterized by a weaker and smaller protection of the DNA duplex against DNase I cleavage. In the ternary complexes, 7–12 nucleotides in the upstream part of the classical footprint are not protected against DNase I, and the hyperreactive sites to DNase I or hydroxyl radicals in this part are also lost. This shortening of the footprint is even more dramatic in the binary complexes. Furthermore, these footprints are already established at low concentrations of enzyme. The DNase I footprint of the ternary complex formed at Ter2 was quantified at different concentrations of RT and at a unique concentration of stabilizing substrate close to the corresponding Michaelis constant. Half-protection of the duplex around the primer terminus is observed at 2 nM RT. This value is close to the apparent dissociation constant of the enzyme from the E-Dn and E-Dn-dNTP complexes reported in the accompanying article (60). Tight complexes with characteristic short footprints are therefore formed at positions Ter2 and Ter2 +1. These footprints are also characterized by unusual sites that are hyperreactive to hydroxyl radicals generated by the Fe-RNase H complex (positions −13 to −11). These hyperreactive sites reflect an atypical positioning of the RNase H domain on the DNA duplex. We interpret these footprints to mean that the binary and ternary complexes formed at Ter2 are different than the ones formed at sites that display processive synthesis.

We then wondered if, during the elongation process at Ter2, these incomplete binary and ternary complexes would isomer-
ize into the classical ternary complex (characterized by a canonical positioning of the RNase H domain) or could bypass this complex and be directly engaged into the elongation step. These two possibilities are presented in Scheme I. The stars indicate the complexes classically observed at positions of processive synthesis.

The effect of increasing amounts of stabilizing nucleotide on the footprints of ternary complexes performed at WT Ter2, probably answers this question. We observed that the atypical positioning of the RT RNase H domain on the DNA duplex is lost and converted into a canonical one when the concentration of incoming dCTP is increased. However, the concentration of nucleotide necessary to restore a canonical positioning of the RNase H domain is much higher than the $K_D^{(dNTP)}$ measured by the kinetic methods. Furthermore, the percentage of hydroxyl radical cleavage never reaches the maximum observed at processive positions. These two observations suggest that the parameters that limit the velocity of the elongation process at Ter2 are different from the parameters that limit the formation of the conventional ternary complex $E-D_n-dNTP^*$. In other words, at WT Ter2, one intermediate of elongation would be a ternary complex characterized by an atypical positioning of the RT RNase H domain. At concentrations of RT and dNTP close to their respective $K_D$, this complex would be able to elongate the primer strand without isomerizing into the canonical ternary complex (path 2). At very high concentrations of RT and dNTP, the elongation scheme could however proceed through the canonical ternary complex (path 1).

Both kinetic and footprinting studies confirm the negative role of the compressed DNA minor groove in the termination process. In our previous studies, we have shown that the narrowing of the DNA minor groove is the structural parameter responsible for termination at Ter2 (30). Kinetic studies have also shown that the mutations that widen the DNA minor groove and abolish termination at Ter2 restore the characteristics of processive elongation at this site. The footprinting studies presented here corroborate this finding. The ternary complex formed at Ter2 on the m-C2 and m-C12 duplexes shows all of the footprinting characteristics of an elongation complex formed at a position of processive synthesis. However, the binary complexes formed on these duplexes do not show all of these characteristics. The positioning of the RNase H domain is atypical as indicated by the presence of sites that are hyper-reactive to hydroxyl radicals generated by the Fe-RNase H complex. This observation suggests that the mutations m-C2 and m-C12, although they are associated with the disappearance of termination products during "long run" assays, do not restore all of the footprinting features that are characteristic of a highly processive synthesis. It is possible that the repetitions of the TG dinucleotide, present in these mutated sequences, disfavor the formation of a classical binary complex but do not perturb the formation of the classical ternary complex.

Both kinetic and footprinting studies have also been performed at various positions around WT Ter2. These studies define a common window around Ter2 where an atypical elongation process occurs. This window is very narrow (positions 4934–4936) and is located six nucleotides downstream of the positions where the width of the DNA minor groove is minimal (positions 4929–4931) (30). According to cross-linking experiments and crystal structures of the ternary complex, the crucial amino acid whose side chain faces the minor groove at position –6 is Gln$^{258}$ of the p66 subunit. This amino acid belongs to the minor groove binding tract. Its mutation to alanine results in a large decrease in enzyme processivity. However, the correlation existing between the negative effects exerted either by the Q258A mutation or by of the A$\rightarrow$T mutation must be taken with caution, since the mutation causes a higher rate of dissociation of the enzyme from the duplex (49), whereas the A$\rightarrow$T mutation affects mainly the rate of elongation of the complex (as shown in the preceding article (60)).

As already reported above, the correlation between the kinetic and footprinting data is not always perfect. Another example of this discrepancy is observed at position 4936. At this position, the conformational change leading to a kinetically competent complex or the chemical step itself is still slower than the dissociation of the enzyme from the elongated duplex (Table I of the preceding article (60)). However, the footprints of the binary and the ternary complexes formed at this position are perfectly conventional. In other words, just before and just after the conformational change, the enzyme is well positioned on the double-stranded portion of the duplex. However, it still feels the obstacle exerted by the A$\rightarrow$T stretch during this change or immediately after. Conversely, at position 4930, the initial binary complex reveals a partial mispositioning of the reverse transcriptase, in particular in the upstream region of the duplex. However, kinetic studies have shown that translocation still occurs faster than dissociation after nucleotide-deincorporation. Therefore, the enzyme may be positioned differently on the duplex, depending on the sequence, a characteristic that could account for the multiphasic profiles frequently encountered on other DNA duplexes.

Structural Model of Termination at Ter2—One of the major characteristics of the complexes formed at Ter2 and Ter2 +1, two positions of nonprocessive synthesis, is the atypical positioning of the RNase H domain of the enzyme on the DNA duplex. Some mutations of HIV-1 RT that affect the catalysis of polymerization but are distant from the RNase H catalytic site have already been reported to alter the position and efficiency of RNase H cleavage (50–52). Nevirapine is also responsible for an alteration of the RNase H cleavage specificity on the RNA template resulting in multiple cleavages around the classical cleavage position (53). Furthermore, the distance between the two catalytic sites of a WT enzyme on a DNA/RNA hybrid or a DNA/DNA duplex depends on the structure of these molecules. This distance is 17 base pairs on the duplex and 18 base pairs on the hybrid (15). It can be even larger when RT encounters a RNA template hairpin (54) or in the case of stuttering (55). Finally, complexes formed by HIV-1 RT on DNA/RNA hybrids that cannot be elongated because of a dideoxyprimer terminus are characterized by unusual RNase H cleavage sites on the template (56). These new cleavage sites are observed around position –8 and are blocked after the addition of the next correct but not incorporeable dNTP. These observations imply that the DNA polymerase activity of this enzyme controls the position of the RNase H domain. Conversely, we observed here a correlation at the termination site Ter2 between the atypical positioning of this domain on the DNA and the impressive drop of processivity of the enzyme.

We propose that this lack of processivity results from a "bad" compromise between the positioning of the two catalytic sites of the enzyme on the DNA duplex. The thumb domain of the catalytic subunit, close to the polymerase site, would be the
first element to sense the narrowing of the DNA minor groove. This unfavorable interaction would prevent the formation of the classical binary and ternary complexes. In contrast, the new complexes formed at the termination site would maintain the favorable interactions between the enzyme and the part of the duplex close to the 3'-end of the primer but would lose the specific upstream contacts of the DNA duplex with the enzyme. The elongation process would then require either the extension of the enzyme footprint and the correct positioning of the RNase H domain or another conformational change. Both pathways would present a high activation barrier that could correspond to the rate-limiting step of the whole elongation process.

This model relies on a coupling between the movement of the two catalytic sites of the enzyme, during the elongation process. It is consistent with other kinetic observations and with different studies performed on mutated enzymes or on the structure of RT-DNA complexes. These studies favor a modular behavior of HIV-1 RT, with possible internal motions like the ones already observed for the fingers and thumb of the catalytic subunit (37, 38, 57, 58). DNA synthesis and enzyme translocation require the alternate opening of at least two enzymatic clamps on the DNA, the polymerization clamp and the RNase H clamp (59). A weakening of one of these clamps and/or a distortion of the DNA double helix between these domains probably affects the polymerase activity of the enzyme. Chemical compounds able to affect this coupling at positions of HIV-1 genome other than the central termination sequence could be designed in the future and could become part of a new antiviral strategy.

Acknowledgments—We are very grateful to Dr. Torsten Unge (Uppsala, Sweden) for the steady supply of HIV-1 RT. We thank Dr. Matthias Goette (McGill AIDS Center, Montreal, Canada), Dr. Jaya Singh and Nathalie Andraos (Harvard Medical School), Dr. Geeta Narlikar (Harvard University), and Dr. Dominique Deville-Bonne (Institut Pasteur, Paris, France) for a critical reading of the manuscript. We also thank Geneviève Legat for technical assistance.

REFERENCES

1. Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) Protein Eng. 3, 461–467.
2. Doublié, S., Sawaya, M. R., and Ellenberger, T. (1999) Struct. Fold Des. 7, R31–R35.
3. Jager, J., and Pata, J. D. (1999) Curr. Opin. Struct. Biol. 9, 21–28.
4. Brautigam, C. A., and Steitz, T. A. (1996) Curr. Opin. Struct. Biol. 6, 54–63.
5. Kunkel, T. A., and Wilson, S. H. (1998) Nat. Struct. Biol. 5, 95–99.
6. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) Nature 314, 763–766.
7. Sarafianos, S. G., Das, K., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999) Chem. Biol. 6, R137–R146.
8. Larder, B. A., and Stammers, D. K. (1999) Nat. Struct. Biol. 6, 103–106.
9. Gotte, M., Li, X., and Wainberg, M. A. (1999) Arch. Biochem. Biophys. 365, 199–210.
10. Thrall, S. H., Krebs, R., Wohlr, B. M., Cellai, L., Goody, R. S., and Restle, T. (1998) Biochemistry 37, 13949–13958.
11. Vincent, J. A., Singh, H. A., and Anderson, K. S. (1999) Biochemistry 38, 10978–10985.
12. Luty, J. M., Keith, G., Le Grice, S. F., Ehresmann, B., Ehresmann, C., and Marquet, R. (1998) J. Biol. Chem. 273, 24425–24432.
13. Isel, C., Westhoff, E., Massire, C., Le Grice, S. F., Ehresmann, B., Ehresmann, C., and Marquet, R. (1999) EMBO J. 18, 1038–1048.
14. Lancy, J. M., Isel, C., Keith, G., Le Grice, S. F., Ehresmann, C., Ehresmann, B., and Marquet, R. (2000) J. Biol. Chem. 275, 12306–12312.
15. Gotte, M., Maier, G., Onori, A. M., Cellai, L., Wainberg, M. A., and Heumann, H. (1999) J. Biol. Chem. 274, 11159–11169.
16. Powell, M. D., and Levin, J. G. (1996) J. Biol. Chem. 70, 5288–96.
17. Palaniappan, C., Kim, J. K., Wisniewski, M., Fay, P. J., and Bambara, R. A. (1998) J. Biol. Chem. 273, 3808–3816.
18. Diaz, L., Cristofaro, J. V., and DeStefano, J. J. (2000) Arch. Virol. 145, 1117–1131.
Structures of Complexes Formed by HIV-1 Reverse Transcriptase at a Termination Site of DNA Synthesis
Marc Lavigne, Lucette Polomack and Henri Buc

J. Biol. Chem. 2001, 276:31439-31448.
doi: 10.1074/jbc.M102976200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102976200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 26 of which can be accessed free at
http://www.jbc.org/content/276/33/31439.full.html#ref-list-1