Human immunodeficiency virus, type 1 (HIV-1) reverse transcriptase (RT) terminates plus-strand DNA synthesis at the center of the HIV-1 genome, a process important for HIV-1 infectivity. The central termination sequence contains two termination sites (Ter1 and Ter2) located at the 3'-end of $A_{m}T_{m}$ motifs, and the narrowing of the DNA minor groove generated by these motifs is responsible for termination. Kinetic data associated with the binding of RT and its ability to elongate in vitro various DNA duplexes and triplexes surrounding the Ter2 terminator were analyzed using a simple kinetic scheme. At Ter2, RT still displays a reasonable affinity for the corresponding DNA, but the binding of the next nucleotide and above all its incorporation rate are markedly hampered. Features affecting the width of the minor groove act directly at this last step. The constraint exerted against elongation by the $A_{m}T_{m}$ tract persists at two positions downstream of the terminator.

Termination of (+)-strand synthesis on a central termination sequence (CTS) can be proposed as a new specific target against HIV-1 replication. Mutations of the CTS that abolish termination are responsible for a large decrease of HIV-1 infectivity. An explanation of this effect has recently been proposed at the molecular and cellular levels. One characteristic of replication of lentiviruses is that their (+)-strand synthesis on a central termination site, has been shown to be the structural element responsible for the termination event (4). Therefore, the width of the minor groove of the synthesized DNA is an important parameter for the polymerase activity of HIV-1 RT.

The role of the DNA minor groove has been confirmed by numerous biochemical and structural studies. Processivity of the enzyme is reduced when amino acid side chains entering the DNA minor groove are mutated (5–7). These amino acids are conserved within lentiviruses, and the cluster has been defined as the minor groove binding tract (MGBT) (8). Modifications of bases have also been informative. Replacement of adenines by diaminopurines in the $A_{m}T_{m}$ motifs both enlarge the DNA minor groove within these motifs and abolish termination at their 3'-end (4, 9). Styrene oxide N2-guanine or N6-adenine adducts introduced in the template strand and pointing toward the minor or major groove, respectively, have been shown to block HIV-1 RT downstream of these modifications (10, 11). With both adducts, the profile and efficiency of termination are different between the WT enzyme and enzymes mutated in residues of the MGBT (12). The location of the termination sites and the difference observed between the WT and mutated enzymes both indicate that the residues of the MGBT of HIV-1 RT are sensing the DNA minor groove of the synthesized DNA and that this sensing is important for HIV-1 processivity. Two structures of complexes formed between RT and a DNA/DNA duplex have been obtained (13, 14). Both structures clearly show a bend of the DNA molecule 4–5 nucleotides upstream of the 3'-end of the primer. This bend is associated with an opening of the minor groove that faces the thumb of the catalytic subunit of the enzyme and more precisely helix $\alpha_{1}$, which contains four of the five residues of the MGBT. Furthermore, during these studies, efficient cross-links have been obtained between RTs mutated in a residue of the MGBT (Q258C, G262C, or W266C) and a duplex that contains a guanine modified at position N-2 (facing the minor groove) in the template strand (15). A catalytic complex of HIV-1 RT has been trapped at various positions by these
cross-links, stressing the role of the DNA minor groove in the interaction between HIV-1 RT and the elongated DNA duplex.

Kinetic studies have shown that the mechanism of ordinary elongation by HIV-1 RT is similar to the one proposed for other DNA polymerases (16–19). Using quench-flow techniques and working in excess of DNA over active enzyme, these studies have suggested a mechanism where the catalytic step is preceded by a conformational change of the enzyme. This isomerization is usually rate-limiting for the incorporation of the nucleotide at saturation of this substrate (corresponding rate constant \(k_c\)) (Scheme I).

Classical kinetic assays, measuring the extension of a primer by one nucleotide and initiated by the addition of the corresponding dNTP, generally display a biphasic profile. The first phase, corresponding to the pre-steady state, can generally be fitted with a monoeponential function. Two parameters are obtained from this phase at saturation in dNTP: 1) the burst amplitude, which indicates the quantity of enzyme that has formed a complex ready to elongate the primer before any event of dissociation (this complex is called a "productive" complex) and 2) the exponential constant, corresponding to the polymerization rate equal to \(k_c\), at saturating concentrations of dNTP. This burst is followed by a steady state phase, initially linear with respect to time. The elongation rate measured during this recycling process divided by the concentration of active enzyme gives the value of \(k_{obs}\), which is the constant for the rate-limiting step of the recycling reaction. On DNA duplexes where synthesis is processive, this step corresponds to the dissociation of the enzyme from the elongated duplex, and therefore \(k_{obs} = k_{off} + k_c\).

However, this simple sequential pathway is not always sufficient to account for some specific cases of elongation. Additional complexes have been postulated to describe RNA-primed initiation of reverse transcription (20–22) or pauses of DNA synthesis induced by secondary structures of the template or by modified nucleotides introduced in the DNA template (23–26).

In the present kinetic study, we examined how the sequential scheme (Scheme I) should be modified to explain the kinetic behavior at Ter2 and at vicinal positions. Minimal modifications in this scheme were introduced to take into account the following observations. At Ter2, in conditions that allow a recycling of the enzyme, elongation by one nucleotide is not limited by the dissociation of the enzyme from the elongated duplex (as observed at processive positions) but by a new rate-limiting step that occurs before this dissociation event and after binding of nucleotide. Assays performed in the presence of a trap show that the elongation rate is also slow with respect to the dissociation rate of the enzyme from the nonelongated duplex. Finally, we examined how these kinetic features depend on the width of the minor groove that precedes the Ter2 termination site and on the position of the elongation complex with respect to this site.

**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 are from Amersham Pharmacia Biotech.

**Classical kinetic assays**

Em

**Enzymatic Assays—**Both rapid and slow kinetic assays are based on the quantification, at different times of the reaction, of elongated (termed "productive") and RT. In the kinetic assays performed in an excess of enzyme, elongation is started by the addition of the corresponding incorporating nucleotide (250 or 900 \(\mu\)M dNTP or 250 \(\mu\)M dNTP). At most positions, elongation performed in the presence of dNTP is restricted to a single incorporation of this substrate. Asterisks indicate these positions. On the WT duplexes, there are three positions indicated in boldface type (4934, 4935, and 4936) where the \(k_{obs}\) is significantly lower than the \(k_{obs}\) measured at processive positions: ND, not determined.

**Table I**

| DNA substrate | Position (3′-OH of the primer) | dNTP (250 \(\mu\)M) | ddNTP (250 \(\mu\)M) | ddNTP (900 \(\mu\)M) |
|---------------|-------------------------------|-------------------|-------------------|-------------------|
| WT duplexes  |                               |                   |                   |                   |
| 4929*        | 0.28                          | 0.22              | 0.19              |
| 4930         | 0.15                          | 0.08              |
| 4931         | 0.26                          | 0.17              |
| 4932         | 0.11                          | 0.08              |
| 4933*        | 0.14                          | 0.12              | 0.12              |
| 4934*        | 0.027                         | 0.019             | 0.021             |
| 4935         | 0.026                         | 0.019             | 0.018             |
| 4936         | 0.38                          | 0.23              | 0.14              |
| 4937*        | 0.17                          | 0.12              |
| 4938         | 0.11                          | 0.06              |
| 4939         | 0.56                          | 0.26              | 0.23              |
| 4940*        | 0.64                          | 0.41              | 0.28              |
| 4941*        | 0.16                          | 0.13              | 0.2               |
| m-C2 duplex  | 0.3                           | 0.17              | 0.18              |
| m-C12 duplex | 0.001                         | ND                | ND                |
| WT triplex   |                               |                   |                   |                   |

HIV-1 RT was generously given by T. Unge (27). Two different batches of RT were used in this study. For each batch, the percentage of active enzyme was measured on an extended PG5/D22 duplex and following an active site method described in Ref. 16.

Duplexes were prepared according to the same protocol. One of the two oligonucleotides (primer or template) is labeled at its 5′-end with \(\gamma^{32}P\)ATP and hybridized to the complementary strand, at a 2:1 ratio (cold-labeled). Hybridization is performed by incubating the duplex for 5 min at 75 °C, followed by a slow decrease in temperature, in a 100 mM Tris-HCl (pH 7.8), 400 mM NaCl, 8% (v/v) polyethylene glycol 6000 solution. Duplexes were always made at concentrations of oligonucleotide at least 20 times higher than their concentrations in the final assay.

Assays were performed at 37 °C and in 50 mM Tris-HCl (pH 7.8), 6 mM MgCl2, 50 mM KCl, and 2 mM dithiothreitol.

**Enzymatic Assays—**Both rapid and slow kinetic assays are based on the quantification, at different times of the reaction, of elongated (\(n + 1\)) and nonelongated (\(n\)) primers. These two forms were separated on a 16% polyacrylamide sequencing gel and quantified using the Phospho-Imaging technique and ImageQuant software.

**Rapid quench experiments** were carried out in a Kintek apparatus (Austin, TX). Experiments were performed by loading the enzyme plus duplex solution in one loop and the dNTP substrate in the second loop. Each reaction was started by rapidly mixing both solutions and quenched with 0.3 mM EDTA after time intervals ranging from 11 ms to several minutes. Before being loaded on the separating gel, the reaction products were ethanol-precipitated in order to overcome problems of migration due to the presence of EDTA.

Slow kinetic assays were performed after a manual mixing of two solutions, preincubated during 5 min at 37 °C. In the measurements of the \(k_{obs}\) of steady state phase (Table I), heparin effect (Fig. 2c), or dGTP effect (Fig. 3), reaction was started by the addition of substrate (dNTP or ddNTP), with or without effector, to a premix of DNA (duplex or triplex) and RT. In the kinetic assays performed in an excess of enzyme, the reaction is started by the addition of enzyme to a premix of DNA and nucleotide.

In both rapid and slow kinetic experiments, performed in an excess of DNA, the rate constant \(k_{obs}\) of the recycling process was always calculated by dividing the velocity of elongation during the linear steady state phase by the concentration of active enzyme (defined on a standard processive hybrid; see above). This calculation assumes that the...
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Fig. 1. Oligonucleotides used in the kinetic study. The sequence surrounding the termination sites Ter1 (position 4925) and Ter2 (position 4934) is presented at the top. Domains C1 and C2 located upstream of these sites and the corresponding Aₜₜ motifs are indicated by brackets and boxes. This sequence has been mutated in domain C2 (mutant m-C2) or in both domains C1 and C2 (mutant m-C12) (3) (mutated nucleotides indicated in boldface type in the corresponding duplex). Below this sequence are presented the oligonucleotides used in the kinetic assays. Primer strands have a common 5′-end (position 4910) and differ by their 3′-end (positions 4929–4941 for WT sequence and 4934 for m-C2 and m-C12 sequences). Template strands are 60-mer oligonucleotides of WT, m-C2, or m-C12 sequences between positions 4905 and 4964. Strands to be displaced are also 60-mer oligonucleotides. They contain an upstream part (between positions 4905 and 4934) that does not hybridize to the template and a downstream part (between positions 4934 and 4974) complementary to the WT and m-C12 templates.

This +1 extension assay was first performed in conditions of large excess of duplexes (130 nM) relative to RT (6 nM). For each time point, the reaction was started by mixing a solution containing RT and the duplex with a concentrated solution of dCTP (250 μM final concentration) and stopped by the addition of EDTA. Reaction times ranged between 11 ms and 180 s. Fig. 2, a and b, shows the kinetics of these extensions. RT clearly displays a different kinetic profile on the WT and mutant duplexes.

The kinetics of primer extension of the m-C2 and m-C12 duplexes conform to the behavior expected for a processive synthesis, as summarized in the introduction (16). They are biphasic and fit well to the following equation: 

\[
\frac{\text{DNAs synthesized}}{t} = A(1 - \exp^{-k_{pol}t}) + Bt, \text{ where } (D_n + 1) \text{ represents the concentration of elongated primer, } A \text{ is the amplitude of the burst, } k_{pol} \text{ is the constant of the pre-steady state exponential phase, and } B \text{ is the slope of the steady state linear phase. Values of burst amplitude, exponential constant, and slope of the linear phase measured in these assays are given in the legend of Fig. 2a. The amplitudes of the burst indicate that 50 and 75% of the present enzyme can form a productive complex at the conditions tested, on the m-C2 and m-C12 duplexes, respectively. The constants of the exponential phase are not significantly different between the two mutants. They are very close to the values of } k_{pol} \text{ measured at processive sites at saturation of dNTP (} k_{pol} = 20 s^{-1} \text{ in Ref. 16). Last, the rate constant } k_{obs} \text{ of the recycling reaction is equal to the slope of the linear steady state phase divided by the concentration of active enzyme (defined on a standard processive hybrid). At processive sites, the recycling process is limited by the dissociation of the enzyme from the elongated duplex. In consequence, on the m-C2 and m-C12 duplexes, } k_{obs} = k_{off}(1-r). \text{ These values (0.4–0.5 s}^{-1} \text{) suggest that RT dissociates slightly faster from these elongated duplexes than from others (0.2 s}^{-1}) \text{.}

On the WT duplex, this biphasic behavior is abolished and a linear release of products occurs immediately after nucleo-
At Ter2, Recycling Elongation Is Not Limited by Dissociation of RT—If the recycling elongation at WT Ter2 is no longer limited by the dissociation of the enzyme from the elongated duplex, one can then specifically perturb this last step and study its consequences on the $k_{\text{obs}}$ constants. This study can be performed at Ter2 on both WT and m-C12 duplexes. Dissociation of RT after elongation is usually disfavored by the addition of the next incorporable deoxynucleotide to the reaction mixture. This effect has been observed when following the incorporation of a deoxynucleotide in the presence of the deoxynucleotide complementary to the next position and when elongation under recycling conditions is limited by $k_{\text{off}}$ (16, 28). The corresponding complex has a longer residence time and is thought to mimic the $E^* - D_{n+1} \cdot dNTP$ ternary complex formed in the sequential reaction pathway.

We analyzed this effect on WT and m-C12 DNA duplexes made with primers that terminate at Ter2. Their elongation was performed with ddCTP in the presence and absence of dGTP (Fig. 3). On the m-C12 duplex, extension is indeed limited by the dissociation of the enzyme at position 4935, since the corresponding value of $k_{\text{obs}}$ is decreased 14 times in the presence of dGTP. By contrast, a less than 2-fold decrease is observed on the WT duplex. Therefore, during a recycling phase, elongation at WT Ter2 is not limited by the dissociation of the enzyme from the elongated duplex but by a step occurring during the polymerization pathway.

**Heparin Challenge—**Heparin, a trap of unbound polymerases, was used to challenge primer extension performed on the WT and m-C12 duplexes (Fig. 4). After formation of the enzyme-duplex complexes, in conditions of excess of duplex (200 nM) over RT (5 nM), heparin was added at the same time as the nucleotide substrate dCTP. The effect of the competitor on the elongation of the two duplexes is clearly different. For the m-C12 duplex (and for m-C2 as well; data not shown), the amplitude of the burst is not affected by inhibition.
Fig. 4. Effect of heparin on the elongation at Ter2 of WT and m-C12 duplexes by HIV-1 RT and dCTP. This experiment was performed at 37 °C by mixing manually a premix solution of duplex (WT and m-C12 k4934/k60, 200 nM, labeled primer) and RT (5 nM) at a solution of dCTP (250 μM) that either contains or does not include heparin (120 μg/ml). At different times, the reaction was terminated by the addition of formamide 95%, EDTA 50 mM, and the n and n+1 forms of the primers were separated and quantified. The quantity of elongated (n + 1) primer was plotted versus the time of the reaction (in seconds). Triangles and circles correspond to values obtained on WT and m-C12 duplexes, respectively. Open symbols and dashed lines, heparin is added at the same time as dCTP; filled symbols and continuous lines, no heparin.

The presence of heparin in the assay. The enzyme-DNA binary complexes formed at m-C12 Ter2 are therefore resistant to heparin and fully committed toward synthesis during the first turnover. This result also implies that the steps that engage these binary complexes into the elongation process are faster than the dissociation of this complex (k_{off(0)} in Scheme I). On the other hand, all complexes formed at WT Ter2 between RT and the DNA duplex are heparin-sensitive. If Scheme I still applies, k_{off(0)} must be faster than the rate-limiting step k_{obs(1)} and all of the binary complexes formed at WT Ter2 can be considered as “slow isomerizing” complexes.

Substrate Effect—The dependence of the rate constant k_{obs(1)} on the dCTP concentration was analyzed at WT Ter2. It follows a simple binding law as shown in Fig. 5. At the plateau, k_{obs(1)} is equal to 0.05 s^{-1}, and the corresponding dissociation constant K_{D(dCTP)} is about 150 μM. This value is much higher than the value K_{D(dNTP)} which characterizes, at positions of processive synthesis, the dependence of the burst rate k_{pol} on nucleotide concentration. This constant corresponds to the binding of dNTP on E-D_n in Scheme I and is reported to be on the order of 4–10 μM (16). Therefore, at Ter2, the rate-limiting step occurs after substrate binding (which is poor) and before dissociation of the enzyme from the elongated duplex.

Kinetic Analysis at WT Ter2 Using an Excess of Enzyme over Duplex—All experiments reported up to now can be interpreted though a simple kinetic scheme, requiring only the sequential binding to the enzyme of the duplex DNA and of the deoxyribo nucleotide substrate. The rate-limiting step occurs after this last binding step and before dissociation of the enzyme from the elongated duplex (Scheme II).

In this model, the role of the enzyme and the duplex are perfectly symmetrical. We can therefore check its relevance by working in excess of enzyme over duplex. Indeed, at WT Ter2, there is no longer any rationale to use the converse situation (excess of duplex), since the information usually provided by a biphasic profile is lost at this position. Scheme II represents an ordinary pathway where product formation (corresponding to the last two species in this scheme) is limited by a step between the ternary complex E-D_n dNTP and the binary complex E-D_{n+1}. The rate of this limiting step is called k_{ls}. Furthermore, we assume that the binding steps leading to the binary complex (dissociation constant K_{D(DNTP)}) and to the ternary complex E-D_n dNTP are fast with respect to this polymerization step. Formation of the elongation products (P) occurs in a single turn over and is given by the following:

$$V = \frac{d[P]}{dt} = \frac{d[E \cdot D_n \cdot dNTP]}{dt} = k_{ls} [E \cdot D_n \cdot dNTP]$$

(Eq. 1)

The concentration of this species is obtained via the two fast preequilibria.

$$[E \cdot D_n \cdot dNTP] = \frac{[E \cdot D_{n+1} \cdot dNTP]}{K_{D(dNTP)}}$$

(Eq. 2)

In consequence, it is related to [D]_T, the total concentration of DNA, by the following.
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\[
[E_D, dNTP] = \frac{[D_T] - [P]}{1 + K_{D,dNTP}K_{D,dNTP}K_{D,E}} [dNTP] [E] [dNTP] (Eq. 3)
\]

When this value is incorporated in Equation 1, product accumulation is shown to proceed via an exponential law characterized by a single time constant, \( \tau E [dNTP] \).

\[
[P](t) = [D_T](1 - e^{-t/\tau}) (Eq. 4)
\]

with

\[
\tau = \frac{k_1[E][dNTP]}{K_{D,dNTP}K_{D,E} + [E][dNTP] + K_{D,dNTP}} (Eq. 5)
\]

At constant substrate concentration, the rate \( \tau^{-1} \) should then follow a Michaelis-Menten behavior as the enzyme concentration \([E]\) is increased. The maximal rate \( k_{max} \) is related to the thermodynamic constant \( k_{th} \) through the following equation.

\[
k_{max} = k_{th} K_{D,dNTP} [dNTP] (Eq. 6)
\]

The apparent Michaelis constant \( K_m(E) \) is related to the dissociation constant \( K_{D,dNTP}(E) \) through the following equation.

\[
K_m(E) = K_{D,E} K_{D,dNTP} [dNTP] (Eq. 7)
\]

This last constant characterizes the dissociation of the pooled complexes \( E-D_n \) and \( E-D_{n-1}-dNTP \) into the free components \( E, D_n \), and \( dNTP \).

Elongation assays were started by the addition of an excess of RT to a premix of duplex \( D_n \) and dCTP. As predicted from Equations 4 and 5, the formation of elongated primer \( D_{n+1} \) follows an exponential function with a plateau and a time constant \( \tau \). These assays are performed at different concentrations of RT, and the \( \tau \) values are plotted versus the inverse of concentration of RT. These plots are called "\( \tau \) plots." This methodology is very close to the one developed by W. McClure in order to study the kinetics of abortive initiation by the *Escherichia coli* RNA polymerase (29, 30).

Data were obtained by this methodology at WT Ter2. They are displayed in Fig. 6, a and b, and in Table II. Three conclusions emerge. First, at all enzyme concentrations tested, a single exponential suffices to fit the time course of product formation (Fig. 6a). This is a test of the homogeneity of population of the kinetically competent complexes bound at Ter2. They all have to go over similar activation barriers (probably the same ones) during the process of primer extension. Second, at the substrate concentration tested, the rate constant \( k_{max} \) (0.017 s\(^{-1}\)) is not very different from the previous rate, \( k_{obs} \), measured in excess of DNA duplex (0.027 s\(^{-1}\)). Using Equation (6) and the value previously determined for \( K_{D,dNTP} \), the \( k_{max} \) value yields a value for \( k_{th} \) of 0.027 s\(^{-1}\). Finally, the apparent Michaelis constant related to enzyme binding is in the nanomolar range (\( K_m(E) = 1.33 \pm 0.23 \) nM). Using Equation 7, this leads to a dissociation constant for the initial binary complex \( E-D_n \) of 3.6 nM, well in the range of ordinary dissociation constants for the binding of RT to DNA-DNA duplexes.

In summary, all complexes engaged into elongation at WT Ter2 appear to conform to the simple sequential scheme given above, and the main constants are shown in Scheme III.

At the termination site Ter2, binding of HIV-1 RT to the duplex is similar to other processive sites, whereas subsequent binding of dCTP is disfavored. However, the major effect is observed at the rate-limiting step, which corresponds to a very slow conversion into product. The corresponding limiting step is 800 times slower than the conformational change that precedes the chemistry step at a processive position.

Modulation of the Strength of the Terminator—Using the same methodology as the one described above, we analyzed how the kinetics of elongation at Ter2 were affected either by changes of the minor groove in the C2 domain or by the requirement of strand displacement on the kinetics of elongation at Ter2.
First, a widening of the groove width can be obtained by mutating the corresponding sequence (mutant m-C2). As observed with the WT duplex, the formation of elongated primer $D_{n+1}$ with the m-C2 duplex fits a monoeXponential function (Fig. 7a). The corresponding time constant $\tau$ follows a Michaelis-Menten behavior as the enzyme concentration is increased (Fig. 7b). The maximal observed rate is $0.13 \text{s}^{-1}$, a value 10 times higher than the one measured on the WT duplex. However, it is also 100 times smaller than the $k_{\text{pol}}$ measured on the same duplex during the pre-steady state phase in conditions of excess DNA. This large difference can be explained by the existence of another limiting step during elongation of the m-C2 duplex. This step would occur during the formation of the $E-D_n$ complex, and its rate would be very close to the rate of dissociation from the elongated duplex. In consequence, on assays performed with an excess of DNA over enzyme and started by the addition of substrate, this other limiting step would not prevent the existence of a burst and would not affect the rate constant $k_{\text{obs}}$ of the steady state phase. On assays performed in conditions of an excess of enzyme over DNA, $k^\text{max}$ would correspond to the rate of this rate-limiting step.

Conversely, we looked at the effect of distamycin on the elongation at WT Ter2. This drug is known to have a good affinity for $A_T$ motifs like the one present upstream of Ter2. A $+1$ extension assay was performed at position 4934 on a WT duplex, in conditions of an excess of enzyme over DNA and in the presence of distamycin (25 nM). The corresponding $\tau$ plot, presented in Fig. 8, fits again a linear relation. Values of $k^\text{max}$ and $K^\text{m}$ obtained from this fit show that the major parameter affected by the presence of distamycin is again $k^\text{max}$. Its value is about 8 times smaller than the one measured in the absence of the drug.

Since we did not measure the values of $K^\text{dNTP}$, at m-C2 Ter2 and at WT Ter2 in the presence of distamycin, we cannot estimate the corresponding values of $k^c$ in these conditions. However, at a constant substrate concentration, the overall activation barrier is reflected by the following.

$$K_{\text{DEB}} = 3.6 \text{nM}$$
$$K_{\text{DGdCTP}} = 150 \text{pM}$$

$K_{\text{DGdCTP}}$ and $K_{\text{DEB}}$ are defined in Scheme III.

![Fig. 7. Elongation of a m-C2 duplex at position Ter2 by HIV-1 RT and dCTP in excess of RT over DNA. +1 extension assays were performed on the m-C2 k4934/k60 duplex under similar conditions as the assays performed on the WT homologous duplex (see legend to Fig. 6). Fig. 7, a and b, represent respectively the kinetic profiles of $E-T_m$ and the derived $\tau$ plot. Values of $k_{\text{max}}$, $K^\text{m}$ and $k_{\text{max}}/K^\text{m}$ estimated from the $\tau$ plot are $0.132 \pm 0.015 \text{s}^{-1}$, $1 \pm 0.3 \text{nm}$, and $13.1 \pm 2.4 \times 10^{-6} \text{s}^{-1}$, respectively.](http://www.jbc.org/)

This ratio is increased by a factor of 10 when the minor groove in C2 is enlarged (as observed on the m-C2) and decreased by a factor of 2 when this groove is occupied by distamycin.

Finally, because strand displacement conditions increase the efficiency of termination, we also performed $+1$ extension assays on a WT Ter2 triplex, in conditions of excess of enzyme over DNA. Fig. 9 shows the corresponding $\tau$ plot. This plot allows a reasonable estimate of $k^\text{max}$ and of $k^\text{obs}/K^\text{m}$ (decreased, respectively, by a factor of 9 and 5 with regard to the WT duplex). Control experiments were performed on the m-C2.
profile was fitted to a monoexponential function, and the \( K_m \) values obtained from these fits were plotted versus the inverse of RT concentration and fitted to a linear function. The fit of the \( K_m \) versus the inverse of RT concentration and fitted to a linear function. This plot and the values of \( K_m, K_{	ext{max}}, \) and \( k_{	ext{max}}/K_m \) estimated from this plot are 0.0043 \( \pm \) 0.0003 s\(^{-1}\), 0.75 \( \pm \) 0.18 nM, and 5.7 \( \pm \) 1 \( \times \) 10\(^{-6}\) s\(^{-1}\)M\(^{-1}\), respectively.

**Values of \( k_{	ext{obs}} \) Measured during the Steady State Phase at Various Positions around WT Ter2**—The kinetic studies presented above revealed that the polymerization rate \( k_{	ext{obs}} \) measured at Ter2 is 740 times lower than polymerization rates measured at processive positions. We wondered whether this very large effect is focused on the Ter2 site or is also observed at vicinal positions. For this purpose, +1 extension assays were performed at positions 4929–4941, with an excess of duplex over enzyme, and the corresponding values of \( k_{	ext{obs}} \) were calculated. Assays were performed using ddNTP instead of dNTP as incorporated substrate, in order to avoid extensions of more than one nucleotide. Furthermore, two different concentrations of ddNTP (250 and 900 \( \mu \)M) were used to investigate the possible stabilizing effect of this nucleotide in case it is complementary to the next position of the template. Finally, whenever possible (positions 4929, 4933, 4934, 4937, 4940, and 4941), the values of \( k_{	ext{obs}} \) corresponding to the incorporation of ddNTP and ddNTP (at the same concentration of 250 \( \mu \)M) were compared.

The values of \( k_{	ext{obs}} \) measured at these different positions on the WT sequence as well as at position 4934 on the m-C2 and m-C12 sequences are presented in Table I. A significant decrease (about 10-fold) of these values is observed at positions 4934, 4935, and 4936 only. This change is independent of the concentration of substrate used in the assay. At Ter2, it does not depend on the nature of the incorporated nucleotide (ddNTP or dNTP). Therefore, extension at the two positions immediately downstream of Ter2 is probably limited, as at Ter2, by a step distinct from the dissociation of the enzyme from the elongated duplex. We propose that this limiting step is similar to the one defined at Ter2.

The self-consistency of our data was furthermore checked by measuring, under the present conditions of excess DNA, the turnover number on a WT triplex at Ter2. This value was found to be 30 times lower than on the corresponding duplex. It is very close to the \( k_{	ext{max}} \) value measured in assays performed with an excess of enzyme over DNA (0.001 s\(^{-1}\)) instead of 0.0019 s\(^{-1}\).

**DISCUSSION**

The main results arising from these kinetic studies are quite simple. At the termination site Ter2, the elongation of the primer strand by one nucleotide is not prevented by a poor binding of the enzyme to the relevant DNA substrate, but by a more difficult association of the nucleotide substrate to the binary complex and, above all, by a very slow elongation rate by the ternary complex \( E-D_n \) dNTP. This effect is lost when the DNA minor groove located upstream of the termination site is enlarged by mutagenesis of the corresponding \( A_{\text{Tm}} \) tract. On the other hand, this effect is enhanced when the minor groove is occupied by distamycin or when the enzyme must also unwind a double-stranded DNA template, downstream of the polymerization site.

**Termination Can Be Described by a Simple Sequential Elongation Scheme**—The first remarkable aspect of these results is that the simple sequential scheme initially proposed to describe elongation by different DNA polymerases is sufficient to explain the kinetics of elongation by HIV-1 RT at Ter2. Results obtained in excess of enzyme over DNA are consistent with the ones obtained in the converse situation. The kinetics of +1 elongation of WT and m-C2 triplexes, at position Ter2, by HIV-1 RT and dCTP. A \( \tau \) plot is shown. The WT k4934/k60 duplex (0.1 nM), incubated with distamycin (25 nM) was elongated at 37 °C by dCTP (250 \( \mu \)M) using various concentrations of RT (enzyme added at the beginning of the reaction). Each +1 extension profile was fitted to a monoeponential function, and the \( \tau \) values obtained from these fits were plotted versus the inverse of RT concentration and fitted to a linear function. This \( \tau \) plot and the values of \( K_m, K_{	ext{max}}, k_{	ext{max}}/K_m \), estimated from this plot are 0.0043 \( \pm \) 0.0003 s\(^{-1}\), 0.75 \( \pm \) 0.18 nM, and 5.7 \( \pm \) 1 \( \times \) 10\(^{-6}\) s\(^{-1}\)M\(^{-1}\), respectively.

**FIG. 8.** Effect of distamycin on the elongation of a WT duplex, at position Ter2, by HIV-1 RT and dCTP. A \( \tau \) plot is shown. The WT k4934/k60 duplex (0.1 nM), incubated with distamycin (25 nM) was elongated at 37 °C by dCTP (250 \( \mu \)M) using various concentrations of RT (enzyme added at the beginning of the reaction). Each +1 extension profile was fitted to a monoeponential function, and the \( \tau \) values obtained from these fits were plotted versus the inverse of RT concentration and fitted to a linear function. This \( \tau \) plot and the values of \( K_m, K_{	ext{max}}, k_{	ext{max}}/K_m \), estimated from this plot are 0.0043 \( \pm \) 0.0003 s\(^{-1}\), 0.75 \( \pm \) 0.18 nM, and 5.7 \( \pm \) 1 \( \times \) 10\(^{-6}\) s\(^{-1}\)M\(^{-1}\), respectively.

**FIG. 9.** Elongation of WT and m-C2 triplexes, at position Ter2, by HIV-1 RT and dCTP, in excess of RT over DNA. A \( \tau \) plot is shown. Triplexes were obtained after annealing of the duplexes (WT and m-C2 k4934/k60) with a 60-mer oligonucleotide complementary to the single-stranded part of the duplex (see Fig. 1). The WT and m-C2 triplexes (0.1 nM) were elongated at 37 °C by dCTP (250 \( \mu \)M) and various concentrations of RT (enzyme added at the beginning of the reaction). Each +1 extension profile was fitted to a monoeponential function, and the \( \tau \) values obtained from these fits were plotted versus the inverse of RT concentration and fitted to a linear function. The fit of the corresponding \( \tau \) plots give values of \( k_{	ext{max}} \) (0.0019 \( \pm \) 0.0002 and 0.035 \( \pm \) 0.006 s\(^{-1}\)), \( K_m \) (0.75 \( \pm \) 0.85 and 1.95 \( \pm \) 1.59 nM) and \( k_{	ext{max}}/K_m \) (2.6 \( \pm \) 2.6 and 1.78 \( \pm \) 1.18 s\(^{-1}\)M\(^{-1}\)) for the WT and m-C2 triplexes, respectively.
extension at Ter2 performed with an excess of enzyme over DNA can always be interpreted by one single exponential function, a result that argues in favor of a unique kinetic pathway for all bound enzyme species.

This uniqueness contrasts with the branched pathways proposed to explain elongation at other nonprocessive sites, like the ones induced by secondary structures of the template or by modified nucleotides introduced in the DNA template (23–26). At these sites, a double exponential function is necessary to fit the initial phase of elongation, in +1 extension assays performed under conditions of excess of DNA over enzyme. This double exponential fit of the initial burst has also been observed during studies on the RNA-primed initiation of reverse transcription (20–22) and on the extension of a DNA/DNA duplex (31). These different studies have proposed the existence of different binding modes of RT to the duplex. One binding mode would lead to the formation of a kinetically competent or productive complex which is characterized by a fast polymerization rate. Another binding mode would lead to the formation of a slow complex, which has to isomerize to the productive complex. This isomerization is attributed to the slow polymerization rate and the amplitudes associated with each polymerization rate (fast and slow) yielding the percentage of productive and slow complexes.

The simplicity of the formalism accounting for our results at WT Ter2 is due to the very slow maximal rate of the limiting step $k_{\text{on}}$. Even if several conformers exist in the populations of binary (E-D$_n$) and ternary (E-D$_n$-dNTP) complexes, reequilibration between these populations is rapid with respect to $k_{\text{on}}$. It is then interesting to estimate the overall effect exerted by the A$_n$T$_m$ tract on the processivity of HIV-1 RT. This can be assessed by comparing at WT Ter2 and at one given concentration of dNTP (250 $\mu$M) the probability to elongate $k_{\text{max}}$ ($k_{\text{max}}$ + $k_{\text{off}}$) versus the probability to dissociate $k_{\text{off}}$($k_{\text{on}}$ + $k_{\text{max}}$). As a result, more than 10 encounters (inverse of the probability to elongate) are necessary for synthesis to proceed. If one assumes a constant $k_{\text{off}}$, then the presence of distamycin increases this number to more than 50 encounters, and the presence of a strand to be displaced downstream of the elongation site increases this value to more than 100 encounters. Together with the sensitivity of the binary complexes to heparin challenge, these calculations indicate that most encounters between the enzyme and the template at Ter2 abort before primer extension.

It is also possible to quantify the negative effect exerted by the A$_n$T$_m$ tract located upstream of Ter2, by comparing the thermodynamic constants measured here at each step of the sequential pathway with similar constants determined at a position where synthesis is processive (e.g., in Ref. 16). At Ter2, the value of $K_{\text{dGTP}}$ is around 3.6 nM, which is very close to the value measured at a processive position (5 nM). Binding of HIV-1 RT to the Ter2 duplex is therefore only slightly favored over binding to a processive duplex ($6G_T \sim -0.2$ kcal/mol). The value of $K_{\text{dGTP}}$, at Ter2 is equal to 150 $\mu$M, indicating that the second step of the sequential scheme is disfavored by 2.17 kcal/mol. Finally, $k_{\text{on}}$ is equal to 0.027 nM$^{-1}$, which is 740 times lower than $k_{\text{on}}$ at a processive site (20 s$^{-1}$) and corresponds to a major penalty of this process (4 kcal/mol). On the whole, the penalty is on the order of 6 kcal/mol.

**Same Kinetic Steps Affected at Ter2 by the Compression of the Minor Groove and by the Constraint of DNA Unwinding**—The second important result obtained in this study is that the main kinetic steps affected during the process of termination at WT Ter2 are always located after the formation of the ternary complex and before the dissociation of the enzyme from the elongated product. Indeed, both modifications in the access of the minor groove of the synthesized DNA (by mutagenesis or binding of distamycin) and the constraint of unwinding the DNA template downstream of the elongation site affect mainly these steps of the sequential scheme. Similarly, pauses observed on RNA and due to the formation of RNA secondary structures downstream of the catalytic site are generally associated with a large decrease of the burst amplitude and with the formation of complexes characterized by good binding affinities and by a slower isomerization into kinetically competent species (corresponding $k_{\text{on}}$ is 1000 times lower) (23). The effect of distamycin on elongation at Ter2 can also be compared with the effects observed during polymerization on a DNA duplex containing cisplatin modifications (25). Again, the major parameter affected by cisplatin modifications is a polymerization rate (“slow” rate = 0.06 s$^{-1}$). On the other hand, pauses of HIV-1 RT (WT or mutated forms affected in their processivity) are not always associated with modifications of the same steps of the polymerization process. In particular, mutations in the MGBT domain, responsible for a decrease of processivity of HIV-1 RT, lead to a decrease of the burst amplitude and to an increase of the dissociation rates of the mutated enzyme from DNA duplexes (5–8, 32). In the case of Ter2, we did not directly measure the rate of dissociation of the enzyme at the termination site. However, the $k_{\text{obs}}$ of the rate-limiting step measured during the +1 extension assay performed at the previous position (4933) can be used to estimate the $k_{\text{obs}}$ at Ter2 (position 4934). As shown in Table I, this value is very close to the value of $k_{\text{off}}$ measured at a processive site (0.2 s$^{-1}$ in Ref. 16). We conclude that the introduction of mutations in the MGBT motif has a more drastic effect than the narrowing of the minor groove where its side chains interact. They prevent the formation of stable initial complexes, while a decreased accessibility of the same groove to the WT thumb essentially prevents the formation of complexes that can quickly isomerize into a productive species.

Finally, we observed that the A$_n$T$_m$ tract, which disfavors so strongly the elongation rate at Ter2 (position 4934), continues to exert its negative effect at the two following positions (4935 and 4936). This phenomenon is indicated by the values of $k_{\text{obs}}$ measured at these positions, which are strikingly lower than the expected values of dissociation rates. At these positions and because of the sequence, the incorporated ddGTP could also play the role of stabilizing nucleotide. However, the low values of $k_{\text{obs}}$ measured at positions 4935 and 4936 do not change significantly when the concentration of ddGTP is increased. Therefore, at these positions, elongation in recycling conditions is also limited by a step different than dissociation from the elongated duplex. Surprisingly, no decrease of the $k_{\text{obs}}$ is observed at the positions located immediately upstream of Ter2. Even if there is a decrease of $k_{\text{on}}$ at these positions, its value is still lower than (or on the order of) the local dissociation rates.

The restriction imposed at Ter2 and at the two vicinal positions on further elongation is therefore extremely severe.

**Open Questions**—These kinetic studies did not allow to define which step is precisely slowed down at Ter2. If the kinetic pathway between the intermediates E-D$_n$ dNTP and E-D$_n$-dNTP is the same at Ter2 as at processive positions, then this slower step could be the isomerization of the ternary complex into an activated ternary complex and/or the chemical step that follows this isomerization. The high value of the dissociation constant associated with dCTP binding already suggests that the substrate binding site is not correctly folded in the E-D$_n$ dNTP ternary complex. In consequence, an isomerization of this complex would be required to reach the kinetically competent complex and would correspond to the rate-limiting step. However, it is also possible that the ternary complex E-D$_n$-dNTP formed
at Ter2 follows a different kinetic path to generate the elongation product. Furthermore, although the same simplified sequential pathway can account for the elongation mechanism at positions where synthesis is processive or distributive (Ter2), it would be misleading to assume that the structure of the postulated intermediates will be roughly identical. The use of footprinting techniques, reported in the companion paper (33), helps to specify the nature of the interactions involved in these transient intermediates.

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