Changes to the HIV Long Terminal Repeat and to HIV Integrase Differentially Impact HIV Integrase Assembly, Activity, and the Binding of Strand Transfer Inhibitors

Received for publication, June 14, 2007, and in revised form, August 20, 2007. Published, JBC Papers in Press, August 21, 2007, DOI 10.1074/jbc.M704935200

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Human immunodeficiency virus (HIV) integrase enzyme is required for the integration of viral DNA into the host cell chromosome. Integrase complex assembly and subsequent strand transfer catalysis are mediated by specific interactions between integrase and bases at the end of the viral long terminal repeat (LTR). The strand transfer reaction can be blocked by the action of small molecule inhibitors, thought to bind in the vicinity of the LTR. Integrase catalysis and high affinity inhibitor binding.

The virus-encoded IN protein catalyzes two essential activities in the viral life cycle, 3′-processing and strand transfer. The 3′-processing reaction cleaves off the final two bases (5′-GT dinucleotide) from the 3′ ends of the viral LTR. The strand transfer activity catalyzes the concerted insertion of the two viral 3′ ends into the host genome with a 5-bp separation. To accomplish this, IN simultaneously positions the two 3′-hydroxyls of the LTRs for nucleophilic attack onto the phosphodiester bonds of the genomic DNA (4). In vitro, both reactions are catalyzed by divalent magnesium or manganese, although magnesium is thought to be the actual metal cofactor in cells (5).

Suitable in vitro systems for studying these processes have been described, in which the full-length protein is minimally required to direct both 3′-processing and strand transfer (6, 7). In these systems, IN is typically allowed to form an in situ complex with a model DNA substrate mimicking a viral LTR end, which then undergoes 3′-processing. The product of the 3′-processing reaction remains bound to IN, forming a strand transfer IN-DNA complex, which is then set up for subsequent strand transfer with a target DNA. In practice, the same synthetic double-stranded oligonucleotides that mimic the U3 and U5 termini of retroviral DNA are often used for both steps in the same reaction, and the products are then separated by gel electrophoresis and quantified by imaging software. The minimal complex thought to be capable of catalyzing strand transfer in vitro is a dimer, although in cells concerted integration of both ends is thought to proceed through a tetrameric complex (8–10). Concerted integration has also been described in vitro (11–15).

The assembly of IN onto the viral LTR stimulates the high affinity binding of strand transfer inhibitors, suggesting that the binding pocket for these inhibitors is derived from a specific structural arrangement of the nucleoprotein complex (16–18). Moreover, such strand transfer inhibitors have been shown to bind in a manner that competes with target DNA for binding to the IN complex (18). However, no x-ray crystal structures are available yet on the specific arrangement of the LTR at the HIV IN active site, although a number of structural models have been proposed (19) based on structural and mechanistic simi-
larities to related INs (20–24) and to other related enzymes such as RNase H, Escherichia coli exonuclease, MuA transposase, Tn5 transposase, and the RuvC-Holliday junction resolvase (25, 26).

The objective of this study was to determine the individual contributions of each of the four terminal bases at the 5’ end of the pre-processed U5 LTR on HIV IN assembly, specific strand transfer activity, and strand transfer inhibitor binding, under conditions where assembly and strand transfer activity were decoupled. Next, we sought to understand if these processes were affected by mutations in the IN protein which confer resistance to IN strand transfer inhibitors. These studies indicate that the 5’-GTCA bases play important and differential roles in strand transfer complex assembly, strand transfer catalysis, and high affinity inhibitor binding. These studies also confirm a central role for Gln148 in assembly, strand transfer catalysis, and inhibitor binding.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Compound 1 and [3H]compound 1 (specific activity 28.6 cpm/μmol, 0.96 × 106 cpm/μl) were prepared by the Medicinal Chemistry Department, Bristol-Myers Squibb. Oligonucleotides were prepared by Sigma Genosys (Woodlands, TX). Scintillation proximity beads were from Amersham Biosciences. The d-spacer and aminopurine monomers were from Glen Research, Sterling, VA. Oligonucleotides were prepared as follows: 0.15 ml of bead-DNA complexes, 2.25 ml of SPA buffer. The proper amount of each IN was prepared (5 μl of IN complexes with 10 μl of TE, 50 mM NaCl. The beads were washed four additional times with TE, 50 mM NaCl, each time centrifuging to remove unbound viral LTR DNA. The final pellet was resuspended in 0.2 ml of PBS and stored at 4 °C before use. Enzyme complexes for 80 strand transfer reactions were prepared as follows: 0.15 ml of bead-DNA complexes, 2.25 ml of SPA buffer (13.3 mM dithiothreitol, 32 mM MOPS, pH 7.0, 0.067% Nonidet P-40, 6.4% polyethylene glycol, 25.6 mM MgCl2, 12.8% (v/v) Me2SO, and 100 mM NaCl), and IN (37 μg of WT, 88 μg of N155H, and 3600, Corning Glass). After 10 min of incubation at 37 °C, 10 μl of the plus strand DNA (96 μM in TE) and 30 μl of the minus strand DNA (96 μM in TE) were combined with 145 μl of TE and annealed at 80 °C for 4 min and then allowed to slowly cool to 20 °C over a 60-min period.

**Determination of Strand Transfer Activity and Inhibition**—The in vitro activities of purified INs complexed with the various duplex LTRs were measured through a scintillation proximity assay (SPA). In a first step, the viral LTR duplexes are prepared by annealing individual oligonucleotides. The viral (donor) LTR DNA is then attached, via a 5’-biotin linker on the plus strand, to streptavidin-coated SPA PVT beads (Amersham Biosciences) as follows. SPA PVT beads (10 mg) were suspended in 0.2 ml of PBS. The suspension was then centrifuged at <5000 × g for 15 min. The supernatant was removed, and the pellet was resuspended with 0.2 ml of PBS, 0.85 mM NaCl, and 21 μl of 12 μM duplex HIV LTR DNA. The sequences of the duplexes are as follows, except for the variations in the underlined bases which are noted in Tables 1–4: plus strand, 5’-biotin-ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA; minus strand, 5’-ACTGCTAGAGATTTTCCACACTGACTAAAAG. The LTR DNA was allowed to bind for 60 min at room temperature with gentle rocking, after which time 0.8 ml of TE was added. The mixture was then centrifuged at <5000 × g and resuspended in 0.8 ml of TE, 50 mM NaCl. The beads were washed four additional times with TE, 50 mM NaCl, each time centrifuging to remove unbound viral LTR DNA. The final pellet was resuspended in 0.2 ml of PBS and stored at 4 °C before use. Enzyme complexes for 80 strand transfer reactions were prepared as follows: 0.15 ml of bead-DNA complexes, 2.25 ml of SPA buffer (13.3 mM dithiothreitol, 32 mM MOPS, pH 7.0, 0.067% Nonidet P-40, 6.4% polyethylene glycol, 25.6 mM MgCl2, 12.8% (v/v) Me2SO, and 100 mM NaCl), and IN (37 μg of WT, 88 μg of N155H, and 3600, Corning Glass). After 10 min of incubation at 37 °C, 10 μl of the plus strand DNA (96 μM in TE) and 30 μl of the minus strand DNA (96 μM in TE) were combined with 145 μl of TE and annealed at 80 °C for 4 min and then allowed to slowly cool to 20 °C over a 60-min period.

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were allowed to stand overnight before reading on a Topcount (PerkinElmer Life Sciences) scintillation counter. HIV-1 IN complexes were evaluated for inhibitor sensitivity using the SPA assay, except that 10 µl of a 5-fold serial dilution of compound 1 in 25% (v/v) Me₂SO/H₂O was added in the place of 10 µl of 25% (v/v) Me₂SO/H₂O. Data were analyzed using GraphPad Prism version 4.03 (GraphPad Software, San Diego) by fitting to a sigmoidal dose-response curve.

**Determination of Dissociation Constants (K_d) and B_max Values for [3H]Compound 1—**Compound 1 (Fig. 1A) is a specific HIV IN inhibitor, with an IC₅₀ in the in vitro strand transfer reaction of 15 nm. A tritiated version of 1 was used to quantify the binding toward WT and mutant IN enzymes assembled with the various LTRs. Reactions were carried out in white 96-well microtiter plates containing 40 µl of pre-formed IN complexes, prepared as described above. A dilution series of [³H]compound 1 was prepared (in SPA buffer), and 10 µl of each dilution was added to separate wells, in duplicate. Microtiter plates were shaken for 3 h to allow complete binding of 1 to the enzyme-SPA bead complex. Binding was measured on a Topcount scintillation counter. K_d and B_max values were calculated by fitting the data to a one-site binding curve using GraphPad Prism version 4.03.

**Calculation of Relative Strand Transfer Activities, Assembly, and [³H]Compound 1 Binding to WT IN Complexes—**Relative strand transfer activities are expressed as a percent of that observed with complexes assembled using WT IN and the WT LTR. [³H]Compound 1 affinity is expressed as a percentage of the association constant K_a (K_a = 1/K_d) for binding to variant complexes, relative to that observed with complexes assembled using WT enzyme and WT LTR. The relative concentrations of complexes are derived from the maximal binding of [³H]compound 1 (B_max), relative to that of [³H]compound 1 binding to complexes assembled using WT IN and a WT LTR. The relative specific activity is defined as the relative strand transfer activity/relative concentration of complexes × 100.

**Competition between Compound 1 and Target DNA—**Thirty µl of pre-formed IN complexes were added to the wells of a 96-well white microtiter plate. Identical sets of control wells contained 30 µl of SPA beads without IN complexes. Ten µl of a serial dilution of 1 in 25% (v/v) Me₂SO was added to the complexes. Binding of a serial dilution of target DNA was initiated by rapid addition, with shaking, of 10 µl of increasing concentrations (1–29 nM) of [³P]-labeled target DNA in SPA buffer. The total of the binding and the products of the strand transfer reaction with target were taken as the difference in signal between wells containing IN and the control wells (without EDTA quenching) after 4 h at 20°C. The data were fit to the Michaelis-Menten equation to give values of V_max and K_m using GraphPad Prism version 4.03 for Windows. The Michaelis-Menten constants were then used to prepare double-reciprocal plots for easier visualization of the mode of inhibition.

**RESULTS**

**Binding of IN to Strand Transfer Inhibitors and Target DNA Are Mutually Exclusive—**One goal of this study was to delineate the individual contributions of assembly and intrinsic strand transfer activity on the overall strand transfer activities of variant LTR/enzyme combinations. Strand transfer activity can be considered to be the result of two different processes. The first is assembly of a functional enzyme-DNA complex, with more efficient assembly expected to produce greater concentrations of active complexes. The second process is considered to be the intrinsic catalytic activity of the complexes. Typically, IN complexes form in vitro at enzyme concentrations between 100 and 1000 nm. However, the actual concentration of active sites is thought to be far lower than the total concentration of IN enzyme, even accounting for the formation of IN tetramers (8). Therefore, to distinguish assembly from intrinsic activity, we measured the concentrations of functionally active sites (those capable of strand transfer) formed for each LTR/enzyme combination. For this analysis we made use of 1 (Fig. 1A), which inhibits HIV IN strand transfer activity in vitro with an IC₅₀ of 15 nm. Radiolabeled [³H]compound 1 binds reversibly to a WT HIV IN complex assembled in vitro with a WT pre-processed LTR substrate (Fig. 1B) with a K_d of 21 nM (Fig. 1C).

The binding of 1 and target DNA to IN was mutually exclusive, as shown by a Michaelis-Menten analysis of strand transfer catalysis in the presence of increasing concentrations of 1 and target DNA. Inhibition by 1 (Fig. 2) was overcome by increasing the concentration of target DNA. A common y-intercept was obtained, indicating that 1 inhibits strand transfer in a manner that is competitive with target DNA, as had been suggested previously (18, 27) for a diketo acid inhibitor. The x intercept provides the −K_i for inhibition by 1. This value is 17 nm, which is in good agreement with the dissociation constant noted above for binding of radiolabeled 1, and similar to the IC₅₀ (15 nm) for inhibition of strand transfer activity.

When complexes derived from 330 nm WT IN were allowed to react with increasing concentrations of [³P]-labeled target DNA to form strand transfer products, the maximal strand transfer product, obtained at saturating concentrations of target DNA, was 2.0 ± 0.4 nm (n = 5). Alternatively, when the
same complexes were allowed to bind $^{[3]H}$compound 1, maximal binding was 4.0 ± 1.0 nM (n = 5). The concentration of active sites capable of entering into strand transfer was about half that of the concentration of sites capable of binding to $^{[3]H}$compound 1. Thus, under the strand transfer conditions of this study, IN acts in a stoichiometric fashion, as the turnover of target DNA was less than 1. A detailed study of the other catalytic activity of IN, i.e. the 3'-processing function (28), concluded that that function also behaved in a single turnover fashion. Therefore, for the purposes of this study, the normalized strand transfer activity for any given LTR/IN combination is calculated as the concentration of observed strand transfer products divided by the maximal concentration of sites capable of binding $^{[3]H}$compound 1.

Effect of Changes to the Viral LTR on Strand Transfer Activity and Inhibitor Binding—We surveyed the effects of changes to the end of the US LTR (Fig. 1B) on strand transfer activity and on the binding of $^{[3]H}$compound 1 to WT IN complexes. Single base substitutions or 2 base extensions of A$^{-2}$ (Table 1, rows 1–5) did not significantly reduce either strand transfer activity (29–31) or $^{[3]H}$compound 1 binding. By comparison, replacement of A$^{-2}$ by iododeoxyuridine reportedly reduced strand transfer activity by 40% (32). A blunt-ended substrate lacking the 5' C$^{-1}$A$^{-2}$ overhang was devoid of both strand transfer and binding activities (Table 1, row 6). This blunt-ended substrate has been examined before, with groups reporting little or no activity (30, 33, 34). Changes to C$^{-1}$ or T$^{1}$ produced only minor changes to either strand transfer activity or $^{[3]H}$compound 1 binding. However, there were two exceptions. First, replacement of C$^{-1}$ by A (Table 1, row 11) was disruptive, as has been previously reported (34). In this case, strand transfer activity and inhibitor binding were reduced to <25 and 50%, respectively. Second, replacement of T$^{1}$ by G (Table 1, row 17) greatly reduced (<25% WT) both strand transfer activity and the binding of $^{[3]H}$compound 1. The consequences of this latter change are discussed in more detail below.

Interestingly, some changes substantially reduce or destroy strand transfer activity but still preserve inhibitor binding. For example, substitution of T$^{1}$ by A (Table 1, row 16) reduced $^{[3]H}$compound 1 binding by only 50%. However, when potential base pairing was restored by simultaneously replacing the cleaved strand 3' A$^{1}$ base to T$^{1}$ (Table 1, row 18), no strand transfer products were observed, as has been reported previously (33). Despite this loss of activity, $^{[3]H}$compound 1 still bound to this complex, albeit with lower affinity (30–50% of WT). Complexes containing a 3'-terminal dideoxy C (ddC, Table 1, row 25), or a deletion of A$^{1}$ (Table 1, row 26), were devoid of strand transfer activity, as would be expected. Notably, IN complexes with substitutions of A$^{1}$ by C, G, T, or ddC, or those assembled with a deletion of A$^{1}$ (Table 1, rows 22–26) were still capable of substantial $^{[3]H}$compound 1 binding. Thus, the catalytic adenosine is dispensable for inhibitor binding. An opposite result was obtained when G$^{2}$ was replaced with A, T, or C (Table 1, rows 19–21). In this case, strand transfer activity was only minimally affected, but all three of these changes caused substantial losses in $^{[3]H}$compound 1 binding (<25% WT).

Overall, these results show that changes to the viral LTR produce one of four different profiles as follows. 1) Changes can be neutral with respect to strand transfer activity and $^{[3]H}$compound 1 binding, i.e. they are without discernible effects, at least under the specific conditions used for these assays (e.g. G$^{-1}$C$^{-1}$A/C; Table 1, row 15). 2) Changes can simultaneously reduce both strand transfer activity and $^{[3]H}$compound 1 binding (e.g. G$^{-1}$A$^{-1}$A/C$^{-1}$; Table 1, row 18). 3) Changes can independently reduce either strand transfer activity (e.g. G$^{-1}$C$^{-1}$A/C) or $^{[3]H}$compound 1 binding (e.g. A$^{-1}$T$^{-1}$A/C; Table 1, row 19), without affecting the other function.

Effects of Base Substitutions and Abase Replacements in the Viral LTR on Relative Specific Strand Transfer Activity and Inhibitor Binding—In an attempt to delineate the effects of specific base changes on strand transfer activity and $^{[3]H}$com-

![Image](https://example.com/image1)

**FIGURE 2.** The mode of inhibition of strand transfer by compound 1 with respect to target DNA was examined by varying the concentration of $^{33}$P target DNA in the presence of increasing concentrations of 1. Concentrations of 1 are as follows: 150 nM, closed squares; 50 nM, open triangles; 16.7 nM, closed triangles; 5.6 nM, diamonds; 1.9 nM, circles.

![Graph](https://example.com/graph1)

**TABLE 1**

| Row LTR | Strand transfer activity | $^{3}$H-Inhibitor 1 binding |
|---------|--------------------------|-----------------------------|
| 1       | GTCA                     | +++                        |
| 2       | GCTT                     | +++                        |
| 3       | GTCG                     | +++                        |
| 4       | GTACA                    | +++                        |
| 5       | GTAG                     | +++                        |
| 6       | GTAC                     | +++                        |
| 7       | GTTA                     | +++                        |
| 8       | GTGT                     | +++                        |
| 9       | GTGC                     | +++                        |
| 10      | GTTG                     | +++                        |
| 11      | GTAA                     | +++                        |
| 12      | GTAG                     | +++                        |
| 13      | GTAT                     | +++                        |
| 14      | GTAC                     | +++                        |
| 15      | GCAC                     | +++                        |
| 16      | GACA                     | +++                        |
| 17      | GACA                     | +++                        |
| 18      | GACA/CT                  | +                          |
| 19      | ATCA                     | +                          |
| 20      | TTCA                     | +                          |
| 21      | CTCA                     | +                          |
| 22      | GTCA/cC                  | +                          |
| 23      | GTCA/CC                  | +                          |
| 24      | GTCA/CT                  | +                          |
| 25      | GTCA/CCdC                | +                          |
| 26      | GTCA/C                   | +                          |

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| TABLE 2 |
| --- |
| Relative strand transfer activities, assembly, and \[^{3}H\]compound 1 binding to WT, N155H, and Q148R IN complexes assembled with LTR variants |
| Strand transfer (ST) activities are expressed as a percent of that observed with complexes assembled using WT IN and WT LTR. Relative \[^{3}H\]compound 1 binding is expressed as a percentage of the association constant (\(K_a\)) for binding to variant complexes, relative to that observed with complexes assembled using WT enzyme and WT LTR. Relative concentrations of complexes are derived from the maximal binding (\(B_{max}\)) of \[^{3}H\]compound 1, relative to that of \[^{3}H\]compound 1 binding to complexes assembled using WT IN and WT LTR complexes. Relative specific activity, relative strand transfer activity/relative concentration of catalytically active complexes that had actually formed (relative \(B_{max}\)) for binding to variant complexes, relative to that observed with complexes assembled using WT enzyme and WT LTR. Relative concentrations of complexes are derived from the maximal binding (\(B_{max}\)) of \[^{3}H\]compound 1, relative to that of \[^{3}H\]compound 1 binding to complexes assembled using WT IN and WT LTR complexes. Relative specific activity, relative strand transfer activity/relative concentration of catalytically active complexes that had actually formed (relative \(B_{max}\)).

| Row | LTR | Relative ST activity | Relative \[^{3}H\]compound 1 affinity | Relative complexes (\(B_{max}\)) | Relative specific ST activity |
| --- | --- | --- | --- | --- | --- |
| 1 | GTCA | 100 | 100 | 100 | 84 |
| 2 | GTG | 109 | 59 | 109 | 90 |
| 3 | GT-G-A | 32 | 37 | 77 | 42 |
| 4 | G-dCA | 70 | 40 | 68 | 33 |
| 5 | dTCA | 10 | 15 | 15 | 67 |
| 6 | TCA | 40 | 8 | 26 | 154 |
| 7 | INTCA | 45 | 62 | 27 | 167 |
| 8 | APCTA | 2 | 24 | 5 | 40 |
| 9 | T-dC-C | 10 | 36 | 54 | 19 |
| 10 | G-d-d-A | 1 | 35 | 36 | 3 |

| TABLE 3 |
| --- |
| Relative strand transfer (ST) activities, assembly, and \[^{3}H\]compound 1 binding to WT IN complexes assembled with LTR variants at C^{-1} |
| Data are as in Table 2, except for base substitutions (underlined) at C^{-1}.

| Row | LTR | Relative ST activity | Relative \[^{3}H\]compound 1 affinity | Relative complexes (\(B_{max}\)) | Relative specific ST activity |
| --- | --- | --- | --- | --- | --- |
| 1 | GTCA | 100 | 100 | 100 | 100 |
| 2 | GTG | 90 | 74 | 66 | 136 |
| 3 | GTG | 86 | 51 | 60 | 143 |
| 4 | GT-dA | 32 | 37 | 77 | 42 |
| 5 | GT-A | 28 | 22 | 54 | 52 |

| TABLE 4 |
| --- |
| Relative strand transfer (ST) activities, assembly, and \[^{3}H\]compound 1 binding to WT IN complexes assembled with LTR variants at T^{1} |
| Data are as in Table 2, except for base substitutions (underlined) at T^{1}.

| Row | LTR | Relative ST activity | Relative \[^{3}H\]compound 1 affinity | Relative complexes (\(B_{max}\)) | Relative specific ST activity |
| --- | --- | --- | --- | --- | --- |
| 1 | GTCA | 100 | 100 | 100 | 100 |
| 2 | G-dCA | 70 | 40 | 68 | 103 |
| 3 | GACA | 62 | 35 | 64 | 97 |
| 4 | GdCA | 5 | 18 | 66 | 8 |
| 5 | GBCA | 70 | 28 | 61 | 115 |

compound 1 binding, we carried out studies on 5’ LTR variants with abasic sites at each of the last four bases of the minus strand (Tables 2–4). These abasic changes were engineered by replacing the natural deoxyribose sugar with a d-spacer (Glen Research), which contains a stable tetrahydrofuran ring attached to a 3’-hydroxyl. It should be noted that the abasic site does not contain the O1 atom, which would be present if the abasic site had arisen through a glycolytic cleavage from a site originally carrying a base. As in previous experiments, strand transfer activity was monitored for each complex. In addition, \[^{3}H\]compound 1 binding to each complex was further quantified by determining the binding affinity (\(K_a\)) of \[^{3}H\]compound 1 toward each IN complex. Because, in principle, changes to the viral LTR may reduce strand transfer activity because of inefficient formation of active IN complexes, the concentration of active complexes produced from each LTR variant was determined by converting the maximal binding (\(B_{max}\)) of \[^{3}H\]compound 1 into concentrations of bound \[^{3}H\]compound 1. Concentrations of bound 1 were then taken as a measure of the concentration of active complexes. In this way, the mechanism for reductions in strand transfer activity could be assigned to result from either an intrinsic change in the catalytic activity of the IN molecules assembled onto the variant LTRs (relative specific strand transfer activity) or to a reduction in the concentration of catalytically active complexes that had actually formed (relative \(B_{max}\)).

Table 2 illustrates the relative strand transfer activity, relative concentration of active complexes, and relative specific strand transfer activity formed from LTR variants with systematic abasic changes. A detailed look at base substitutions at C^{-1} and T^{1} is shown in Tables 3 and 4, respectively. Of note, replacement of C^{-1} by A (Table 3, row 5) greatly reduced strand transfer activity (28%), due only, in part, to a reduction in the concentration of active complexes (54%). This is essentially the same result as an abasic replacement at C^{-1} (Table 3, row 4). The negative effect on strand transfer activity by a replacement of C^{-1} by A has been reported previously (34). Of note, \[^{3}H\]compound 1 affinity for this C^{-1} to A complex was also greatly reduced (22%). Overall, an abasic site in place of C^{-1}, or substitutions of C^{-1}, reduced the efficiency of assembly by up to 2-fold and also produced up to ~2-fold decreases in specific strand transfer activity. In addition, the inhibitor-binding site is altered such that \[^{3}H\]compound 1 affinity is reduced 2–5-fold relative to WT. Thus, the inhibitor binding data may indicate that C^{-1} plays a base-specific role in inhibitor binding.

Base substitutions to T^{1} are shown in Table 4. When T^{1} was replaced with an abasic spacer (Table 4, row 2) or with A (Table 4, row 3), strand transfer activities were slightly reduced because of reductions in assembly. Specific strand transfer activities were the same as for WT. However, the effects on \[^{3}H\]compound 1 affinity were more pronounced (reduced ~3-fold). A different profile resulted from a replacement of T^{1} by G (Table 4, row 4). In this case there was a very large reduction in both specific strand transfer activity (8% of WT) and \[^{3}H\]compound 1 affinity (18% of WT). On the other hand, assembly was only marginally affected (66%). Thus, the substitution of T^{1} by G produces much larger effects on specific strand transfer activity and inhibitor binding compared with
TABLE 5
Summary of the effects of abasic replacements the 5’ end of the U5 LTR on assembly, relative strand transfer activity, relative specific strand transfer activity, and inhibitor affinity

Strand transfer (ST) activities and [3H]compound 1 binding are relative to those of WT IN assembled onto the WT LTR. ++ +++, >70%; ++ +, 40–69%; ++, 20–39%; +, 10–19%; +/-, 2–9%; −, <2%. * indicates data could not be calculated as there is trace of ST activity, but no detectable [3H]compound 1 binding.

| Integrase | No change | 1ST | 2ST | 1A | 2A |
|-----------|-----------|-----|-----|----|----|
| Assembly  |           |     |     |    |    |
| WT        | ++ +      | +   | +   | ++ | +  |
| N155H     | ++ +      | +   | +   | ++ | +  |
| Q148R     | +         | -   | +/− | +/−| +  |

| Relative ST activity | | | |
|----------------------|---|-----|
| WT                   | ++ + | + |
| N155H                | ++ + | + |
| Q148R                | +   | -   |

| Relative specific ST activity | |
|-------------------------------|-----|
| WT                            | ++ + |
| N155H                        | ++ + |
| Q148R                        | +   |

| Affinity | |
|----------|-----|
| WT       | ++ + |
| N155H    | +/−  |
| Q148R    | +/−  |

Effects of Simultaneous Changes to Both the Viral LTR and IN on Strand Transfer Activity and Inhibitor Binding—Replication of HIV in the presence of strand transfer inhibitors selects for substitutions near the active site for strand transfer (37, 38). We characterized the effects of two of these amino acid substitutions on strand transfer activity and inhibitor binding in the context of changes to the four terminal bases of the viral LTR. The first is a change of asparagine 155 to histidine (N155H), previously reported to confer resistance to an azanaphthyridine strand transfer inhibitor in a rhesus macaque model (39), and recently observed in the clinic with elvitegravir (40). The second is a change of glutamine 148 to arginine (Q148R). Both substitutions have been reported to contribute to resistance to two different IN inhibitors in the clinic (41). Results of using the N155H and Q148R IN proteins with the WT LTR are shown in Table 2 and summarized, along with the data for WT IN, in Table 5. Data in Table 2 are given as percentages relative to WT IN assembled onto a WT LTR. Assembly of N155H was the same as WT, whereas Q148R had an ~4-fold assembly defect (Table 2, row 1). N155H and Q148R complexes (with a WT LTR) had slightly reduced specific strand transfer activities as compared with the WT enzyme. N155H and Q148R affinities for [3H]compound 1 were reduced to 10 and 3%, respectively, as expected, demonstrating that resistance is because of reduced inhibitor affinity.

Next we evaluated the combined effects of the mutant INs in the context of each of the LTR variants. Certain changes to the LTR were without additional effects. For example, deletion of A−2 (Table 2, row 2) produced no further defects to N155H and Q148R assembly, strand transfer activity, or [3H]compound 1 affinity. Although abasic C−1 or T1 sites (Table 2, rows 3 and 4) reduced assembly (~2-fold), it was to a similar level as induced in the WT IN background. [3H]Compound 1 affinities, already reduced in this background by ~6-fold versus WT IN, were not further reduced by abasic C−1 or T1 sites. By contrast, defects in the N155H/abasic G2 combination (Table 2, row 5) were significantly magnified compared with either N155H complexes prepared with a WT LTR (Table 2, row 1) or WT IN complexes prepared with an abasic G2 (Table 2, row 5). Strand transfer...
activity and inhibitor affinity were reduced by >50-fold, and assembly was reduced to \( \leq 5\% \), the lower limit of detection. When \( G^2 \) was replaced by inosine (Table 2, row 7), specific strand transfer activity and \( [^{3}H] \) compound 1 affinity were restored to at least that of N155H assembled with the WT LTR, but assembly, although improved, remained defective (17%). Overall, alterations at \( G^2 \) which destroy \( G^2:C^2 \) base pairing are more detrimental to assembly/strand transfer activity/binding in the context of N155H IN, as compared with those in the context of WT IN, and more disruptive than abasic sites at \( C^{-1} \) and \( T^1 \). By comparison, inosine/C2 has an intermediate effect on assembly/strand transfer activity/binding, as compared with no disruption (WT LTR) or complete disruption (abasic G2). As noted previously, inosine was also able to replace \( G^2 \) in the context of WT IN with the same profile, i.e. similarly reduced efficiency of complex formation (27%), and near-WT strand transfer activity and \( [^{3}H] \) compound 1 binding. Thus, inosine appears to significantly restore the structure of the inhibitor binding site to that formed with a WT LTR, presumably by partially restoring base pairing at the 2-position.

With the exception of an \( A^{-2} \) deletion, most of the variant LTRs introduced substantial assembly and specific strand transfer defects to the Q148R active complex. For example, although a Q148R complex with an abasic \( T^1 \) site (Table 2, row 4) does form, its assembly is 3-fold poorer than for a WT IN complex assembled with the same abasic \( T^1 \), and it was nearly devoid of any specific strand transfer activity. When Q148R was assembled with the abasic \( C^{-1} \) (Table 2, row 3), only a very low amount of complex was detected by both \( [^{3}H] \) compound 1 binding and strand transfer activity. Based on a lower level for detection of assembly to be \( \sim 5\% \), we estimate that \( C^{-1} \) reduces Q148R assembly by 6-fold, on top of the 4-fold assembly defect associated with Q148R alone. The low concentration of this complex did not allow for the direct determination of \( [^{3}H] \) compound 1 affinity. To circumvent this limitation, strand transfer activity was inhibited with a nonradioabeled version of 1. The resulting IC50 was compared with that for inhibition of the WT complex by 1. The resulting relative IC50 values indicate that 1 has \( \sim 2\% \) of the WT affinity toward this combination, similar to the loss of affinity measured for the WT LTR-Q148R complex. Overall, these results indicate that abasic \( T^1 \) and \( C^{-1} \) sites produce synergistic defects in the assembly and specific strand transfer activity of Q148R, without further reducing already highly attenuated 1 affinity.

Introduction of an abasic G2 to Q148R complexes was the most disruptive combination studied (Table 2, row 5). This combination had no detectable catalytic activity or inhibitor binding. As noted previously, inosine partially rescues the assembly, strand transfer activity, and inhibitor binding defects conferred by an abasic G2 in both WT and N155H backgrounds. However, inosine fails to rescue the defects of an abasic G2 in the Q148R background (Table 2, row 7). Thus, Q148R imposes even greater constraints on complex assembly with LTRs containing alterations to \( C^{-1}, T^1, \) and \( G^2 \), as compared with either WT or N155H IN, and does not form or forms only trace amounts of active complexes on LTR variants containing changes at these bases.

**FIGURE 3.** Magnesium dependence of \( [^{3}H] \) compound 1 binding and strand transfer activity. A, assembled IN complexes were resuspended in magnesium-free buffer, and binding of \( [^{3}H] \) compound 1 was initiated by the addition of magnesium to the indicated concentrations. Association constants (\( K_a \)) were determined at each magnesium concentration. The data from four independent experiments were analyzed as a percentage of the \( K_a \) observed at 100 mM magnesium (maximal). WT, circles; N155H, squares; and Q148R, triangles. B, magnesium dependence of strand transfer activity. Assembled IN complexes were resuspended in buffer containing the indicated magnesium concentrations. Strand transfer was initiated by the addition of \( ^{33}P \) target DNA. The data are the average of three experiments.

**Magnesium Ion Dependence of Strand Transfer Activity and Binding Affinity of \( [^{3}H] \) Compound 1 for the Different Enzyme-LTR Complexes**—Molecular modeling studies point to a possible role for a flexible loop (comprised of amino acids 140–149 of HIV IN) in the catalytic mechanism that places a magnesium ion at the active site (23). Because Q148R IN had a greatly reduced binding affinity for \( [^{3}H] \) compound 1, we reasoned that inhibitor binding might display an altered magnesium dependence versus WT. As shown in Fig. 3A, Q148R IN complexes require at least 10-fold higher magnesium ion concentrations to affect maximal inhibitor binding, as compared with WT complexes. By comparison, the maximal binding of N155H had only a slightly altered magnesium ion dependence versus WT. Additionally, we examined the ability of the enzymes to function at their maximal level under varying magnesium conditions. The extent of strand transfer activity did increase for all three enzymes with increasing magnesium concentrations, peaking at around 20 mM Mg2+ for the WT and N155H enzymes. Q148R showed a shift compared with the other enzymes, as it takes \( \sim 10\)-fold higher magnesium concentrations for Q148R to function at its maximal level. These data
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indicate that the compound binding and strand transfer defects of Q148R are because of an altered affinity of magnesium for the active site of the complex. In turn, this suggests a structural change to the positioning of the ligand sphere around the magnesium atoms.

Interestingly, maximal inhibitor binding for the WT enzyme requires ~10-fold higher magnesium concentrations than that required for strand transfer (30 versus 3 ms; compare peaks in Fig. 3, A versus B). This suggests that magnesium operates stochiometrically to assist in driving the equilibrium from the unbound to the inhibitor-bound state. However, because assembled complexes bind to target DNA even in the absence of magnesium (28), magnesium is only required catalytically to effect strand transfer and likely shuttles between strand transfer active sites.

**DISCUSSION**

Using conditions where assembly and strand transfer activity were decoupled, we have probed the individual contributions of each of the four terminal 5'-bases at the U5 LTR with regard to assembly of a functional complex, strand transfer activity, and small molecule inhibitor binding. This was also explored in the context of two IN variants containing substitutions that are known to encode reduced sensitivity to these small molecule inhibitors. Only pre-processed LTRs were used to form IN complexes under experimental conditions where strand transfer activity occurred only when double-stranded target DNA was added. This approach eliminated confounding effects on strand transfer activity or inhibitor binding, which might result from the in situ 3'-processing of unprocessed substrates.

In these studies we used INs containing a histidine tag. In our hands, WT IN lacking a His tag binds to [3H]compound 1 with the same affinity as to IN containing a His tag, and produces similar levels of strand transfer products in vitro (data not shown). Also, losses in affinity of [3H]compound 1 toward the resistant INs assembled with a WT LTR (in vitro) mirror the same losses in potency of compound 1 (in cell culture) toward viruses containing only these specific amino acid changes (data not shown). These data indicate that the His tag does not alter proper IN assembly, strand transfer activity, or inhibitor binding.

Initial studies proved that under these assay conditions, IN acts as a single turnover enzyme with respect to a double-stranded DNA target, to produce strand transfer products. This point has not been rigorously proven before, although the single turnover nature of the 3'-processing function of IN has been demonstrated (28). Next we showed that the binding of the strand transfer inhibitor used in this study is mutually exclusive with respect to that of the double-stranded target DNA. This conclusion was previously reached by analysis of the displacement of binding of target DNA to an LTR terminated by a deoxyadenosine (18, 27). However, to perform subsequent studies, it was necessary to show that under our assay conditions inhibitor binding was competitive with that of target DNA binding. We also determined that the stoichiometry of strand transfer products to inhibitor binding was <1. Thus, given the single turnover nature of the enzyme, we were able to quantify a specific strand transfer activity for each IN/LTR variant by normalizing strand transfer activity to the concentration of active sites. This approach enabled any defects in complex assembly to be differentiated from those arising from defects in intrinsic strand transfer activity.

There have been many reports detailing the contributions of the terminal bases of the HIV LTR toward its catalytic 3'-processing function. These will be briefly reviewed here. The most extensive investigations have focused on the determinants of 3'-processing and have demonstrated the importance of the terminal 5'-C1A2 dinucleotide (6, 29–31, 35, 42–46). End fraying at the 5'-C1A2 dinucleotide (45, 47) and/or localized distortions (48–50) at the 3' A1 residue are required to permit efficient 3'-processing, and synthetic LTRs bearing non-complementary changes at this end undergo 3'-processing more efficiently than WT (45). By contrast, the sequence requirements for strand transfer activity have been suggested to be more relaxed than for 3'-processing (47), with the notable exception of substitutions to the 3' terminal A1 residue. Nevertheless, certain bases at the 5’ end of the LTR are known to have important functions in strand transfer complex assembly, strand transfer catalysis, and the binding of strand transfer inhibitors.

A fair amount of information has been reported concerning the function of the 5'-C1A2 dinucleotide overhang after 3'-processing. It has been shown to be important for integrase-LTR complex stability (51) based on its interaction with a loop (amino acids 140–149) (25, 52–53) of the IN core domain (46), in particular with residue Gln148 (54). Gln148, in turn, has been shown to specifically cross-link with C1 (55) and possibly with A2 (42). In line with these observations, a Q148L mutant reportedly bound the product of disintegration, i.e. essentially the strand transfer-competent intermediate, less well than WT (56), and the virus was defective for replication (57). Molecular modeling and crystallographic data have suggested that this loop is flexible (52, 58–61), implying it has a role in catalysis beyond that in IN/DNA assembly. In fact, replacement of Gly140 (62), or a combination of Gly140 and Gly149 by alanines (58), or Gln148 by alanine (55), was reported to reduce or eliminate 3'-processing, strand transfer, and disintegration activities.

The 5' C1 overhang is reported to be required for efficient use of a pre-processed substrate in the strand transfer reaction. Changing C1 to a T barely produced any end joining products (34). Non-3' processed substrates, with substitutions of C1 by A, T, and G, had reduced strand transfer activities (54). Also, a construct with deletions of both the terminal A2 residue and the C1 base, leaving only an intact sugar phosphate on T1, underwent normal 3'-processing but lacked strand transfer activity. Thus, the published data suggests that C1 plays a role in transitioning the 3'-processing complex to one subsequently competent for strand transfer (54). Because C1 appears to directly interact with Gln148 (54), it is plausible that this transition- ing role for C1 may actually be a structural rearrangement facilitated by the aforementioned flexible loop.

In addition to its roles in assembly and catalysis, the flexible loop reportedly plays a key role in the binding of specific strand transfer inhibitors. For example, the G140S mutant has reduced affinity for the strand transfer inhibitor L-731,988 (62).
Also, Lee and Robinson (55) reported that both G149S and Q148A were 11- and 22-fold resistant, respectively, to the strand transfer inhibitor L-731,988, although certain recently described dihydroxythiophene strand transfer inhibitors appear to bind differently and are not resistant to Q148K (63). A direct interaction between Gln148 and strand transfer inhibitors has been suggested by several other studies. An x-ray crystallographic study found that the strand transfer inhibitor, 5-CITEP, is in close proximity to Gln148 (64). Selection for resistance to the strand transfer inhibitor S-1360 resulted in the change of Gln148 to Lys (65). Most importantly, clinical data indicate that Q148R/Q148K/Q148H substitutions along with G140S/G140A reduced the sensitivity of the virus to raltegravir (66).

Molecular dynamics simulation (67) of 5-CITEP binding to the resistance conferring mutant T66/M154I indicated that there were major differences in the conformational flexibility of the 138–149 region compared with the WT IN, suggesting an intimate involvement of loop placement with respect to inhibitor binding. Finally, a recent IN-inhibitor binding model concluded that inhibitor binding is in close proximity to the flexible loop and that inhibitor binding impedes loop mobility and hence catalytic activity (68). Thus, the flexible loop and Gln148 in particular are important for not only assembly and catalysis but also for strand transfer inhibitor binding.

Table 5 provides a simplified summary of the data presented in this paper. The key findings of this study, which bear on the inter-relationships between the 5’ end of the LTR and the IN flexible loop, are summarized as follows.

1) Defects in assembly and activity associated with changes to G2, T1, and C1 are magnified in strand transfer inhibitor-resistant backgrounds. This effect is particularly striking in the context of the Q148R mutant. Assembly is poor; strand transfer activity is weak or absent, and inhibitor binding is severely attenuated. With the exception of a deletion of A2, which did not further magnify the defects associated with Q148R, abasic replacements poorly assembled and had either very low (T1, 3% of WT) or no strand transfer activity (C1 or G2). These results are understandable in the context of the presumed interaction of the flexible loop with the 5’ end of the LTR.

We found that the origin of the strand transfer defects and reduced inhibitor binding to Q148R is because of an altered affinity of magnesium for the active site of the complex. Q148R requires 10-fold higher magnesium concentrations to produce maximal binding relative to WT and N155H INs. Furthermore, compared with WT, the peak strand transfer activity of Q148R was also achieved at 10-fold higher magnesium concentrations. This implies that Gln148 is involved in the affinity of one or both of the bound magnesium ions thought to be present in the active site of the complex. A structural explanation for this result is suggested by the placement of the 140–149 flexible loop in two different crystal structures (52, 53). The structures agree in the placement of two active site residues, Asp64 and Asp116, but each have different loop placements, which alter the positioning of a third active site residue Glu152. It is thought that Glu152 is ligated to the loosely bound magnesium during catalysis, and may be a ligand in the inhibitor-bound state (69). Therefore, we suggest that Q148R reduces the affinity for strand transfer inhibitors by shifting the loop equilibrium away from the state that directs Glu152/magnesium binding toward one that favors the state without bound magnesium. Dynamics simulations indicate that Gln148 adopts a conformation that is dependent on metal ions at the active site (59), and it has been suggested that the conformational change exhibited by the active site upon metal binding is stabilized by the presence of phosphate in the catalytic site (70). By analogy, sufficiently high concentrations of inhibitor likely stabilize the magnesium-bound state, working in opposition to the loop disruption induced by Arg148. We speculate that the introduction of changes to the 5’ terminus further destabilizes magnesium binding, hence accounting for the synergistic impact of these changes on assembly, activity, and inhibitor binding in the context of the Q148R IN.

2) In the N155H background, only minor levels of strand transfer activity, and reduced levels of specific strand transfer activity, are detected in N155H complexes assembled with an abasic G2 (<2%) or a G2 to T change (4%), although significant assembly and activity of WT complexes with these LTR changes were observed. A possible explanation for this result may be that Asn155 is located near the catalytic Asp64 and Glu152 amino acids, and not too distant from the 5’ end of the viral LTR, as suggested through molecular modeling studies (19). It is likely that perturbations at both Asn155 and the G:C base pair destabilize the H-bonding network required for assembly and activity. By contrast, defects in assembly, specific activity, and inhibitor binding induced by changes to C1 and T1 were not further amplified in the context of N155H, as these changes produced 2–3-fold reductions in both assembly and specific strand transfer activities relative to WT, in either background. This suggests that the structural changes introduced by changes at C1 and T1 operate independently of those introduced by N155H, whereas those at G2 are interdependent with N155H.

3) Single base changes to C1 and T1 produce modest (<2-fold) assembly, relative strand transfer activity (2-fold), and inhibitor affinity defects (1–5-fold) to WT IN, whereas deletion of A2–5 has little effect on these properties. Two changes to the 5’ end of the LTR (abasic C1→T1 or an LTR containing an abasic C1 plus a deletion of A2–5; see Table 2, rows 9–10) induce the most severe defects in specific strand transfer activity (reduced 5- and 30-fold, respectively). Taken together, the binding data support the idea that the inhibitor makes either direct or indirect base-specific interactions with both T1 and C1.

4) Substitutions, or an abasic replacement at G2, severely reduce all three IN functions, highlighting the importance of this specific base to the structure of the complex in general and to the inhibitor-binding site in particular. An abasic G2, or replacement of G2 by other bases, produced the largest effect on inhibitor binding (12-fold) of all the variant LTRs. We hypothesize that the reason for the reduced inhibitor binding is because of the loss of a critical base pair between G2 and C2. This base pair is likely the last base pair of the viral terminus in the cleaved complex. For this reason, the G:C base pair likely serves an important role in organizing the active site structure, as suggested by the large effects on assembly that accompany its removal or replacement. In support of the idea of the impor-
tance of the G:C base pair to the active site architecture, we found that the loss of inhibitor binding induced by this abasic G2 change was reversed by substitution with inosine. Inosine is capable of forming two of the three hydrogen bonds of the normal G:C base pair, and the same minor groove binding as guanine, thus indicating that two hydrogen bonds preserve the architecture needed for high affinity inhibitor recognition. However, it is noteworthy that the inosine replacement still has an assembly defect (27% of WT). This indicates that the hydrogen bonding contribution of the guanine amino group is an important contributor to assembly of the nucleoprotein complex. Interestingly, it has been reported that inosine in place of G2 severely retards the rate of 3' -processing (71). Our results would suggest that the reported negative effect of inosine at G2 on 3' -processing is because of poorer assembly, as the assembly defect associated with an abasic G2 site was not corrected by the inosine substitution.

5) Replacement of T1 by G (Table 4) produces a large deleterious effect on specific strand transfer activity, independent of assembly. Inhibitor binding is also reduced 5-fold. Interestingly, inosine rescues the strand transfer defect but does not rescue the binding defect. Possible explanations are that the larger size of the purine versus the pyrimidine base is responsible for these effects, or perhaps the projection of the 2-amino group introduces an inappropriate hydrogen bond to some other part of the active site structure, thus interfering with strand transfer catalysis and inhibitor binding.

Taken together, our results suggest a synergistic relationship associated with simultaneous alterations of at least two of four determinants, i.e. Gln148, C−1, T1, and G2, on complex stability, strand transfer activity, and inhibitor binding, lending further support to the idea that Gln148 and possibly other determinants of the Gly140−Gly149 flexible loop maintain specific interactions with the 5' end of the LTR. A number of unanswered questions remain concerning the exact roles of the viral LTR on assembly, strand transfer activity, and inhibitor binding. In particular, the dynamics of the structural mechanism whereby Gln148, C−1, T1, and G2 exert their affects on these inter-dependent processes requires further structural delineation. Hopefully, a more complete mechanistic picture of these interactions will continue to emerge.

Acknowledgment—We thank Dr. Ying-Kai Wang for helpful comments on the manuscript.

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