Effect of DNA Modifications on DNA Processing by HIV-1 Integrase and Inhibitor Binding

ROLE OF DNA BACKBONE FLEXIBILITY AND AN OPEN CATALYTIC SITE

Received for publication, May 30, 2006, and in revised form, July 13, 2006. Published, JBC Papers in Press, August 30, 2006, DOI 10.1074/jbc.M605101200

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Integration of the viral cDNA into host chromosomes is required for viral replication. Human immunodeficiency virus integrase catalyzes two sequential reactions, 3′-processing (3′-P) and strand transfer (ST). The first integrase inhibitors are undergoing clinical trial, but interactions of inhibitors with integrase and DNA are not well understood in the absence of a co-crystal structure. To increase our understanding of integrase interactions with DNA, we examined integrase catalysis with oligonucleotides containing DNA backbone, base, and groove modifications placed at unique positions surrounding the 3′-processing site. 3′-Processing was blocked with substrates containing constrained sugars and α-anomeric residues, suggesting that integrase requires flexibility of the phosphodiester backbone at the 3′-P site. Of several benzo[a]pyrene 7,8-diol 9,10-epoxide (BaP DE) adducts tested, only the adduct in the minor groove at the 3′-P site inhibited 3′-P, suggesting the importance of the minor groove contacts for 3′-P. ST occurred in the presence of bulky BaP DE DNA adducts attached to the end of the viral DNA suggesting opening of the active site for ST. Position-specific effects of these BaP DE DNA adducts were found for inhibition of integrase by diketo acids. Together, these results demonstrate the importance of DNA structure and specific contacts with the viral DNA processing site for inhibition by integrase inhibitors.

HIV-1 integrase (integrase) catalyzes insertion of cDNA copies of the viral genome into human chromosomes. Integrase binds to the ends (“att” sites) of each viral long terminal repeat (LTR) through sequence-specific recognition of a conserved 5′-CA within the sequence 5′-GCA GT. In the first of two reactions, integrase cleaves the 3′-ends of the viral DNA, releasing the terminal 5′-GT dinucleotide (3′-processing, 3′-P). In the second reaction, the free 3′-hydroxyl of the conserved adenine provides the nucleophile for insertion of the viral cDNA into a chromosome (strand transfer, ST). Gap repair and ligation between the viral and cellular DNA are performed by cellular factors. (For recent reviews and insights on integration, see Refs. 1–5.)

Determination of the molecular interactions between integrase and its DNA substrates (viral and chromosomal DNA) has proven challenging, and a co-crystal of these components remains elusive. Biochemical studies have revealed contact points between the viral DNA and integrase. Integrase has an absolute requirement for the conserved 5′-CA adjoining the 3′-P site (underlined in Fig. 1A). The efficiency of 3′-P is also dramatically decreased by changes to the G immediately 5′ to the conserved CA dinucleotide (6–8). The conserved adenine, substituted by 5-iododeoxyuracil as a photocross-linker, forms a photocross-link to Lys-159 of integrase (9). Residue Lys-159 also contacts the phosphate 5′ to the conserved deoxyadenosine (10). Mutagenesis showed that Tyr-143 and probably Gln-148 interact with the 5′-overhang resulting from 3′-P (9, 11). Moreover, disulfide cross-linking revealed proximity of the conserved amino acid residue 148 (Q148C mutant) to the second (cytosine) base and of residue 246 (E246C mutant) to the seventh (adenine) base from the 5′-end of the lower strand of the U5 LTR (see Fig. 1A) (12, 13).

Interactions between the backbone of the viral DNA and...
Integrase have been sparsely examined. Phosphate ethylation interference was used to locate phosphates that are critical for integrase catalysis (14). Specific DNA backbone contacts required for ST near the insertion site were identified. On the cleaved strand of the viral DNA, the phosphate 5′ to the conserved adenine, as well as the two phosphates on the complementary strand that are closest to the cleavage site, were important for ST. The two phosphates at and following the ST site were important within the target DNA (14). Together, these observations suggest many stabilizing contacts between integrase, the tip of the viral cDNA LTR, and the target DNA. Additional information is required for higher resolution modeling and structure-based design of integrase inhibitors.

Integrase 3′-P and ST can be examined with a simple in vitro assay using recombinant integrase, duplex oligonucleotides derived from the sequence of the last 21 bp of the U5 LTR (Fig. 1A), and a divalent metal cofactor. 3′-P produces a 19-mer product on denaturing sequencing gels as the 5′-GT dinucleotide is cleaved (see Fig. 1D, Ctl + IN lane). In the same assay, ST is achieved by insertion of the processed DNA into another identical duplex, resulting in a ladder of products migrating slower than the substrate DNA in denaturing sequencing gels. Here we present data from experiments using manganese so that our results are comparable with prior studies (7) and to create the most permissive conditions for integrase reactions. The data in Figs. 4–7 were also performed with magnesium, with similar results (data not shown).

The use of synthetic oligonucleotides allows studies of the effects on integrase activity of site-specifically placed DNA modifications. Here we focused on DNA backbone and base modifications surrounding the adenine of the conserved 5′-CA dinucleotide sequence to probe the DNA contacts between integrase and the viral DNA ends. We examined the effect of several DNA backbone modifications on integrase activity. Oligonucleotides containing conformationally constrained sugars attached to the conserved adenine were used to examine the conformational preference for north (north bicyclo[3.1.0]hexane and locked nucleic acid [LNA]) or south (south bicyclo[3.1.0]hexane) oriented sugars (Fig. 1C, see structures). A simple ribose substitution was also examined for comparison to LNA. Additionally, the effects of anomeric inversion on integrase 3′-P and ST were examined using oligonucleotides containing α-anomers around the 3′-P site (Fig. 2A). Substitution of an α-anomer results in a change in the normal 5′→3′ direc-

![FIGURE 1. Inhibition of HIV-1 integrase 3′-P by restriction of sugar puckering. A, sequence of the final 21 bp of the HIV-1 U5 long terminal repeat. The conserved 5′-CA is underlined, and the 3′-P site is indicated by a triangle. B, normal nucleic acids exist in rapid equilibrium between north and south orientations of sugar puckering. The equilibrium tends toward the south for B-DNA and the north for RNA. C, the location (restricted nucleotide in boldface) and structures of the conformationally locked nucleosides are shown within the sequence of the five terminal nucleotides of the HIV LTR. Pymol images in stereo are derived from Protein Data Bank codes 1EK2, 1OF1, and 1I5W. D, representative gels showing the effect of north (n) and south (s) conformationally locked bicyclo[3.1.0]hexane nucleosides, LNA, and adenine ribonucleoside (riboA) at the site of 3′-P compared with normal DNA (Ctl, control). The cleaved ribo-containing oligonucleotide migrated slower than the fully deoxyribo-containing oligonucleotide, and the migration of the ribo-19-mer is noted. IN, integrase.](image-url)
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FIGURE 2. Inhibition of HIV-1 integrase 3′-P by anomeric inversion. A, schematic structure of the last four nucleotides of the “α1” oligonucleotide. The phosphate on the 3′-side of the conserved adenine (the scissile phosphate) is directly linked to the 3′-OH guanosine, resulting in polarity inversion. B, sequences of oligonucleotides containing anomeric inversions. Oligonucleotide directionality is indicated by arrows above the sequences and α notation at the site of polarity inversion. α1 has a 3′–3′ polarity inversion starting with the guanosine of the cleaved dinucleotide. α2 has a 3′–3′ polarity inversion starting with the conserved adenine. α3 has a polarity inversion only for the conserved adenine (3′–3′ followed by 5′–5′ reversion). The 3′-P site is indicated by the triangle. C, representative gel showing the effect of α nucleotides on integrase (IN) 3′-P. Ctl indicates the normal DNA control. All oligonucleotides containing anomeric inversions are annealed to unmodified complementary strands.

MATERIALS AND METHODS

Oligonucleotide Synthesis—All oligonucleotides were derived from the sequence of the last 21 bases of the HIV U5 LTR (Fig. 1A), with the exception of substitutions described. Unmodified, abasic site, and ribose-containing oligonucleotides were commercially synthesized by IDT (Coralville, IA). 7-Deazaadenine and 2-aminopurine containing oligonucleotides were commercially synthesized by Midland Certified Reagent Co., Inc. (Midland, TX). Synthesis for bicyclo[3.1.0]hexane, LNA, α-anomer, and BaP-modified oligonucleotides is described in the Supplemental Material.

All oligonucleotides were further purified on denaturing 20% polyacrylamide gels. Single-stranded oligonucleotides were 5′-labeled using T4 polynucleotide kinase (Invitrogen) with [γ-32P]ATP (Amersham Biosciences) according to the manufacturers’ instructions. Unincorporated nucleotide was removed by mini Quickspin oligo column (Roche Applied Science). The duplex DNA was annealed by addition of an equal concentration of the complementary strand, heating to 95 °C, and slow cooling to room temperature.

Integrase Reactions—Recombinant wild-type HIV-1 integrase was purified from Escherichia coli as described (21) with the addition of 10% glycerol to all buffers. Integrase was incubated with DNA substrates for 1 h at 37 °C. The reaction conditions were 500 nm integrase, 20 nm duplex DNA, 7.5 mM MnCl2, 5 mM NaCl, 14 mM 2-mercaptoethanol, and 20 mM MOPS, pH 7.2. Reactions were quenched by the addition of an equal volume of gel loading dye (formamide containing 1% SDS, 0.25% bromophenol blue and xylene cyanol). Products were separated on 20% polyacrylamide denaturing sequencing gels. Dried gels were visualized using a 445 SI PhosphorImager (Amersham Biosciences). Densitometric analysis was performed using ImageQuant software from Amersham Biosciences.

Schiff Base Cross-linking Assay—The Schiff base cross-linking experiments were performed as described (22). Briefly, oligonucleotides containing uracil at position U3 (annealed to unmodified or adducted lower strands) or L7 (annealed to unmodified or adducted upper strands) were 5′-32P-labeled as described above. After annealing, uracil DNA glycosylase was added to create an abasic site at the uracil position. The abasic site leads to the formation of a Schiff base cross-link between the aldehyde group on the ribose and a nearby integrase lysine. The cross-links were stabilized by addition of 100 μM sodium borohydride (final concentration). The cross-linked integrase-DNA products were separated from the substrate DNA by SDS-PAGE using 16% Tricine gels (Invitrogen).
RESULTS

Effect of Sugar Modifications on Integrase Reactions—The sugar conformation of standard B-DNA exists mainly in C-2'-endo conformation (south or s), whereas the less frequent A-form with a C-3'-endo conformation (north or n) is more typical of RNA (Fig. 1B). Oligonucleotides containing conformationally restricted sugar puckers at the site of integrase 3'-P were used to probe the conformational preferences of integrase during catalysis. Integrase recognizes the conserved 5'-CA in the HIV LTR (underlined in Fig. 1A). Adenosine analogs containing bicyclo[3.1.0]hexane (north or south, n or s), LNA, or ribonucleoside sugars were substituted for the conserved deoxyadenosine (Fig. 1C). The three conformationally constrained sugar modifications prevented integrase 3'-P, as indicated by the lack of 19-mer product (Fig. 1D, lanes 5, 6, and 10). The ribonucleoside permitted 3'-P and ST (Fig. 1D, lane 14), suggesting that the presence of the 2'-O functionality in LNA exhibits minimal interference with integrase catalysis. Note that the cleaved ribo-containing oligonucleotides migrated slightly slower in the gel compared with fully deoxyribo-containing oligonucleotides, but a 20-mer band is present between the full-length and cleaved ribo-containing oligonucleotides, and therefore, we presume the altered migration is because of the extra hydroxyl group. These results show that conformational restrictions at the 3'-P site block 3'-P.

Effect of Anomeric Inversion on Integrase 3'-P—The phosphodiester backbone conformation was further examined by placement of nucleotides containing 3'-anomerically inverted oligonucleotides at and around the site of integrase 3'-P. The normal B-DNA β-anomers connect via 5'-3' phosphodiester linkages. The presence of a single β-anomer requires 3'-3' and 5'-5' internucleotide linkages and a switch in the directionality of the DNA (Fig. 2, A and B) in order to permit Watson-Crick base pairing with an unmodified complementary strand. Substitution of the terminal GT with two α-anomers (Fig. 2, A and B, α1, 3'-3' linkage) permitted a small amount of 3'-P (84% inhibi-
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The addition of a third \( \alpha \)-anomer extending to the conserved adenine completely blocked 3'-P (Fig. 2, B and C, \( \alpha_2 \), 3'–3' linkage). Finally, the presence of a single \( \alpha \)-anomer substitution for the conserved adenine also resulted in a complete block of 3'-P (Fig. 2, B and C, \( \alpha_3 \), 3'–3' then 5'–5' linkages). Together with the results obtained using modified sugars, it is clear that the backbone structure around the 3'-P site is critical for catalysis by integrase.

Use of BaP DE Adducts as Molecular Probes—We next used two types of BaP DE adducts to study the effects of DNA groove occupancy and intercalation on integrase catalysis. Adducts with 10R and 10S configuration at the point of attachment (Fig. 3, C-10 of the hydrocarbon) to the exocyclic 6-amino group of adenine intercalate on the 5'- or 3'-side of the added base, respectively (Figs. 3, A–C, and 4A). The partially saturated ring linked to the adenine protrudes into the major groove and therefore provides major groove bulk that may potentially interact with integrase (Fig. 3, A–C).

Adducts in which the hydrocarbon is attached to the exocyclic N-2 of guanine have the aromatic pyrene ring system located in the minor groove (Fig. 3, D and E) extending toward the 5'- or 3'-end of the added strand for the S and R stereoisomers, respectively (see Fig. 4A). By convention, the adducts will be referred to by their upper and lower strand positions from the viral DNA end. For example, U3 refers to the 3rd base from the end of the upper strand and U3(S) is the isomer with 10S configuration located at the U3 position.

Effect of Intercalating \( dA \) Adducts Attached to the Conserved Adenine on Integrase Reactions—The effect of site-specific DNA intercalators on integrase catalysis was probed using oligonucleotides containing a single BaP DE adduct attached to the conserved adenine (Fig. 3, A–C). NMR structures indicate that these adducts provide some bulk in the major groove and that the hydrocarbon stacks mainly with bases on the unadducted strand (see Fig. 3C from Ref. 17). Adducted DNAs were examined as full-length (Fig. 4A, upper sequences) and pre-cleaved (Fig. 4A, lower sequences, pc) duplex substrates. We showed previously that the lower strand L4(S) minor groove adduct blocks 3'-P and ST (Fig. 4B) (7). In contrast, intercalating BaP DE adducts attached to the conserved adenine (U3) had no affect on 3'-P and a partial inhibition of ST (55 and 60% inhibition, Fig. 4B). A similar effect on ST was observed for pre-cleaved DNA. Complete inhibition of ST by L4(S) adducted DNA, and partial inhibition of ST (58 and 67% inhibition) by the U3 adducted DNAs was observed (Fig. 4B). These results demonstrate that BaP DE adducts linked to the conserved adenine reduce ST without affecting 3'-P.

Importance of Functional Groups on the Conserved Adenine for Integrase Reactions—Because the \( dA \) adducts are attached to the adenine exocyclic 6-amino group, the importance of the conserved adenine base was further evaluated through several base modifications (Fig. 5). 2-Aminopurine, lacking the 6-a-amino group (Fig. 5B), was chosen to evaluate the importance of the point of attachment of the intercalating BaP DE adducts. 2-Aminopurine substitution resulted in no decrease in 3'-P and only a slight decrease in ST (Fig. 5C, 14%). Hence, the exocyclic N-6 group of the conserved adenine is not required for integrase activity. Next, 7-deaza-adenine was chosen because of the possible importance of the adenine N-7 (9). Fig. 5C shows that replacement of the adenine N-7 with carbon caused no change in integrase 3'-P and ST (Fig. 5C). In contrast, substitution of adenosine with an abasic (tetrahydrofuran) site resulted in a partial (67%) inhibition of 3'-P and complete loss of ST (Fig. 5C). It is important to note that this substitution probably affects the backbone structure as well as base pairing. These results indicate that integrase tolerates modifications on the 2 and 6 positions of the conserved adenine but that removal of the base inhibits integrase activity.

**FIGURE 4.** Effect of \( dA \) intercalating and \( dG \) minor groove BaP DE adducts on HIV-1 integrase 3'-P and ST.

* A, sequences of full-length (upper duplexes) and pre-cleaved ("pc", lower duplexes) DNA substrates showing the location of \( dA \) intercalating (vertical bars) and \( dG \) minor groove (horizontal bars) adducts. B, representative gel showing the effect of \( dA \) intercalating and \( dG \) minor groove BaP DE adducts on integrase reactions. Ctl indicates the unadducted DNA control. U3 BaP DE adducts retard electrophoretic migration. IN, integrase.
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Effect of Minor Groove BaP DE Adducts Linked to the 3’-Processed Dinucleotide on Integrase Reactions—We next examined the effect of single trans-opened BaP DE dG adducts attached to the guanine (U2) of the cleaved dinucleotide (Fig. 7A). This position was not addressed in our prior study (7). Therefore, we wished to extend our minor groove footprinting through the end of the DNA. The U2 minor groove adducts slightly inhibited 3’-P (Fig. 7, B and C). Because 3’-P presumably releases the terminal dinucleotide, we were surprised to observe a more pronounced inhibition of ST. To test whether the added dinucleotide could act as an ST inhibitor, we examined the effects of an added dinucleotide (5’-GT containing BaP DE adduct) added to integrate reactions performed with an unadducted (control) DNA substrate. Little inhibition of 3’-P or ST was observed (30% only at 333 μM; data not shown). These results demonstrate that BaP DE adducts on the U2 guanine reduce ST without affecting the efficiency of 3’-P, suggesting the retention of the added dinucleotide within the integrate-DNA complex following 3’-P (see “Discussion”).

Use of BaP DE Adducts to Study Inhibition of Integrase by the Diketo Acid L-708906—Because diketo acid ST inhibitors have been hypothesized to bind at the enzyme-DNA interface at the end of the viral DNA (1, 23, 24) and because the BaP DNA adducts residing near the integrase 3’-P site decreased overall ST, which mimics the effects of DKA, we examined the effect of these BaP DE adducts on inhibition of integrase by the DKA L-708906 (Fig. 8B). However, L-708906 is an ST-selective inhibitor of integrase with normal DNA (25) (Fig. 8, A–C). We observed a marked inhibition of 3’-P for the U2(S) dG-added DNA (Fig. 8, E and F, and Table 1), indicating this adduct increases the ability of L-708906 to act as a 3’-P inhibitor. Inhibition of the remaining ST was unchanged for the U2(S) dG-added DNA (Fig. 8E and Table 1), which is expected as 3’-P releases the adducted dinucleotide (Fig. 8D). In contrast, 3’-P inhibition by L-708906 was unaffected by a U3(S) dA adduct (Fig. 8H and Table 1). Furthermore, we observed an increase in the IC_{50} value for ST by L-708906 in the presence of the U3(S) dA adduct compared with unadducted DNA (Fig. 8H and Table 1), indicating that the U3(S) dA adduct interferes with ST inhibition by L-708906. By contrast, the terminal L1(S) dA adduct has no effect on integrase inhibition by L-708906 (Fig. 8K). Similar results were obtained with magnesium (data not shown). These results demonstrate that the presence of BaP DE adducts at specific positions affect integrase inhibition by L-708906.

Effects of BaP DE Adducts on Inhibition of Integrase by Ma-DKA, L-870810, and L-Chicorinic Acid—Because of the effects of the U3(S) dA and U2(S) dG adducts on inhibition of integrase by L-708906, we extended our studies to another ST-selective DKA inhibitor, Ma-DKA (4, 26). Similar results were observed and are summarized in Table 1. As for L-708906, the U2(S) dG adduct increased 3’-P inhibition by Ma-DKA (Fig. 8B, compare closed symbols), and the U3(S) dA adduct reduced ST inhibition by Ma-DKA (Fig. 8F, compare open symbols).

The naphthyridine carboxamide L-870810 is also an ST-selective inhibitor like DKA's but induces different resistance mutations (27). The U2(S) dG adduct increased inhibition of
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3'-Processing by Integrase Requires DNA Backbone Flexibility

The flexibility of DNA probably plays an important role in enzymatic site-specific recognition and catalysis of DNA modifications by enzymes. For example, thymidine kinase preferentially phosphorylates its substrate thymidine in the south ribose conformation, whereas mammalian cellular DNA polymerase(s) show a preference for the thymidine-5'-triphosphate in the north ribose conformation (30). North-restricted bicyclo[3.1.0]hexane-containing nucleoside triphosphates act as delayed chain terminators of reverse transcriptase when DNA synthesis approaches the A- to B-DNA transition point (31). LNA is RNase-resistant (32), and α-anomic oligodeoxynucleotides are also resistant to endonuclease S1 and exonucleases (calf spleen phosphodiesterase and snake venom phosphodiesterase) (33). Obviously, conformational restriction of the phosphodiester backbone and anomeric inversion impart significant structural effects on nucleic acids.

In our study, both north and south conformationally restricted sugars inhibited integrase 3'-P (Fig. 1). Note that manganese was used for these experiments, which is more "permissive" than magnesium for integrase reactions. Integrase was however able to bind to each duplex DNA containing a conformationally constrained substitution at a level similar to unmodified duplex, as measured by a Schiff base assay (22) (data not shown). Additionally, the ribose 2'-O-substitution of LNA is not inhibitory to integrase 3'-P as cleavage is observed in the presence of a ribonucleotide substitution. The α-anomer-containing duplex oligonucleotides were similarly resistant to integrase cleavage (Fig. 2), whereas binding integrase at a similar

Reduced slightly the inhibition of ST, which is different from the DKAs (compare Fig. 9G with Ma-DKA in Fig. 9F and L-708906 in Fig. 8H). Hence, the naphthyridine carboxamide L-870810 exhibits differences for BaP DE adduct interference compared with the DKAs L-708906 and Ma-DKA.

Finally, l-chicoric acid was chosen for comparison because of the following: 1) a G140S resistance mutation (28) does not overlap with DKA resistance mutations (25), and 2) because l-chicoric acid inhibits both 3'-P and ST (29), indicating a different binding site compared with DKAs. Similar levels of 3'-P and ST inhibition by l-chicoric acid were observed for both U2(S) dG- and U3(S) dA-adducted DNAs compared with unadducted DNA (Fig. 8, D and H). Together, these results demonstrate that BaP DE adducts can reveal site-specific differences in the inhibition sites for integrase inhibitors.
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TABLE 1
IC₅₀ values* for inhibition of integrase by inhibitors in the presence of BaP DE adducted oligonucleotides

| Inhibitor | Reaction | DNA substrate | IC₅₀ | IC₅₀ | IC₅₀ |
|-----------|----------|---------------|------|------|------|
| L-708906  | 3'-P     | U2(S)/21      | >12.3| 0.4  | 5    |
| Ma-DKA    | 3'-P     | ST            | >12.3| 5.2  | >12.3|
| L-870810  | 3'-P     | U2(S)/21      | >12.3| 6    | >12.3|
| L-Chloric acid | 3'-P  | ST            | 0.19 | 0.39 | 0.12 |

* Data were obtained from dose-response curves generated from three independent determinations (see Figs. 8 and 9). IC₅₀ expressed in μM.

FIGURE 8. Effect of BaP DE adducts on inhibition of integrase by the DKA L-708906. A, schematic diagram of integrase reactions with unadducted DNA. B, representative gel showing inhibition of integrase (IN) by L-708906 in the presence of unadducted DNA. C, schematic diagram of integrase reactions with DNA containing the U2(S) dG adduct. This diagram presumes that following 3'-P, the dinucleotide containing the BaP DE adduct is released, and the ST substrate does not contain an adduct. In graphs E, H, and K, 3'-P is indicated by filled symbols and ST by open symbols; unadducted DNA substrates are indicated by triangles and adducted substrates by squares. Note that these symbols are also indicated in the left panels. D, quantification of inhibition of integrase 3'-P by L-708906 with and without a U2(S) dG adduct on the substrate DNA. E, schematic diagram of integrase reactions with DNA containing the U3(S) dA adduct. The ST substrate contains a BaP DE adduct. F, quantification of inhibition of integrase 3'-P by L-708906 with and without a U3(S) dA adduct on the substrate DNA. G, schematic diagram of integrase reactions with DNA containing the L1(S) dA adduct. The ST substrate contains a BaP DE adduct. H, quantification of inhibition of integrase 3'-P with and without a L1(S) dA adduct on the substrate DNA.

nucleosides with constrained sugars and anomeric inversions demonstrate the importance of a flexible DNA backbone for integrase catalysis. Attachment of BaP adducts to the adenine of the conserved 5'-CA at position N-6 (Fig. 4) and removal of N-7 and exocyclic N-6 amino groups still allow 3'-P and ST (Fig. 5), indicating these groups are not required for integrase catalysis. Additionally, substitution of the conserved adenine with an abasic tetrahydrofuran resulted in a 67% inhibition of 3'-P (Fig. 5), both in the presence of manganese and magnesium (data not shown). Although we recognize that adenine must be important for integrase binding and catalysis, the tetrahydrofuran substitution probably also distorts the DNA backbone structure. Previous substitution of the conserved adenine with a propan-1,3-diol residue (abasic hydrocarbon bridge (38–40)) resulting in a loss of 3'-P may in fact be reanalyzed in terms of backbone contacts with integrase. Hence, our results with substitution of

level as unmodified DNA was measured by Schiff base assay (data not shown).

Nucleic acids containing pseudo sugars and anomeric inversion are known to be resistant to exo- and endonucleases (33, 35, 36), but to our knowledge the underlying nuclease resistance mechanism has not been clarified. We propose that these backbone modifications may act by 1) altering the presentation and 2) restricting the flexibility of the scissile phosphate. A slight misalignment of the DNA within the active site of a nuclease could prevent the precise coordination of DNA and metal required for cleavage. Incorporation of an α-anomer into DNA necessitates addition or removal of one carbon atom because of the inverted directionality of the α nucleotide. The resulting misalignment of the scissile phosphate may critically block nuclease cleavage. Restriction of phosphate flexibility propagated from a conformationally constrained sugar could inhibit inversion of the phosphate to achieve the bipyramidal transition state required for in-line attack by a nucleophile. For example, the 3'-hydroxyls of north and south bicyclo[3.1.0]hexane nucleotides are fixed equatorially and axially, respectively. In our case, that translates into a differential positioning of the leaving group connected to the scissile P–O bond without the flexibility of a normal deoxyribose. As the integrase reaction proceeds with inversion of configuration of the phosphate during 3'-P (37), the rigidity of the conformationally locked nucleosides may impose an energy barrier to achieving the pentacovalent intermediate where the nucleophile and leaving group are in the apical positions. In fact, this may be a general nuclease resistance mechanism for oligonucleotides such as LNA that are used in gene therapy and antisense techniques.

Substitutions at the Conserved Adenine of the Viral DNA Still Allow 3'-P and ST—Our studies using oligonucleotides with constrained sugars and anomeric inversions demonstrate the importance of a flexible DNA backbone for integrase catalysis. Attachment of BaP adducts to the adenine of the conserved 5'-CA at position N-6 (Fig. 4) and removal of N-7 and exocyclic N-6 amino groups still allow 3'-P and ST (Fig. 5), indicating these groups are not required for integrase catalysis. Additionally, substitution of the conserved adenine with an abasic tetrahydrofuran resulted in a 67% inhibition of 3'-P (Fig. 5), both in the presence of manganese and magnesium (data not shown). Although we recognize that adenine must be important for integrase binding and catalysis, the tetrahydrofuran substitution probably also distorts the DNA backbone structure. Previous substitution of the conserved adenine with a propan-1,3-diol residue (abasic hydrocarbon bridge (38–40)) resulting in a loss of 3'-P may in fact be reanalyzed in terms of backbone contacts with integrase. Hence, our results with substitution of
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FIGURE 9. Effect of BaP DE adducts on inhibition of integrase by Ma-DKA, L-870810, and L-chicoric acid. A, schematic diagram of integrase reactions with DNA containing the U2(S)-dG minor groove adduct. Presumably following 3'-P, the dinucleotide containing the BaP DE adduct is released. The ST substrate does not contain an adduct. B–D, quantification of inhibition of integrase 3'-P (filled symbols) and ST (open symbols) by Ma-DKA, L-870810, and L-chicoric acid, respectively, with unadducted (triangles) and U2(S) dG-adducted (squares) DNA substrates. E, schematic diagram of integrase reactions with DNA containing the U3(S) dA adduct. The ST substrate contains a BaP DE-dA adduct. F–H, quantification of inhibition of integrase 3'-P (filled symbols) and ST (open symbols) by Ma-DKA, L-870810 and L-chicoric acid, respectively, with unadducted (triangles) and U3(S) dA-adducted (squares) DNA substrates.

the conserved adenine indicate that an intact adenine structure itself is not absolutely required for 3'-P and ST.

Importance of Viral DNA Minor Groove Contacts at the 3'-P Site for Integrase Catalysis—Scanning of the viral LTR minor groove with BaP DE adducts illustrates specific contact regions for integrase activity. We previously examined the effects on integrase reactions of BaP DE adducts linked to the U5 and L4 guanines (7). We also examined the effect of each BaP adduct on integrase activity in the presence of magnesium and obtained similar results (data not shown). The U5(S), U5(S), and L4(S) adducts completely blocked 3'-P, and all stereoisomers at both positions blocked ST (7). We examined the U2 position in the present study to extend minor groove scanning in the vicinity of the 3'-P site. The U2 adduct still allowed efficient 3'-P and only partially reduced ST. Therefore, we suggest that integrase makes specific contacts with the viral LTR minor groove at the 5th and 4th bp from the DNA end, corresponding to 5'-GCAGT-3'. The minor groove closer to the tip of the minor groove for viral DNA binding and 3'-P performed by HIV-1 compared with HTLV-2 integrase reactions.

Moreover, NMR analysis of the terminal 17 bp of the HIV U5 LTR showed the following: 1) that the region near the cleavage site has a distorted minor groove and 2) base stacking between the L4 guanine and U3 adenine exposes the adenine 3'-hydroxyl in the minor groove for 3'-P, aiding in specific recognition by integrase (43). It is possible that the minor groove near the processing site interacts with integrase providing specificity of binding to the active site. The major groove would be exposed to solution during 3'-P, but may be more important during ST for contacts with other integrase subunits or the target DNA.

Tolerance of BaP Adducts at the End of the Viral DNA Indicate That Integrase May Have an “Open” Active Site—The integrase active site may be open because we find that integrase can still function in the presence of U2 dG and U3 dA adducts. An open active site is reasonable because the target DNA needs space to approach the active site for the ST reaction. Release of the 5'-terminal dinucleotide may be required to create this open active site for ST. Although release of the dinucleotide is generally assumed to follow 3'-P, to our knowledge, this has not been shown experimentally. The ~60% loss of ST in the presence of the U2 dG adduct may indicate that the presence of the adduct on the dinucleotide prevents its release. The BaP DE adduct could bind to the enzyme active site following 3'-P and cause the dinucleotide to “stick” in the active site pocket (Fig. 10E), thereby prohibiting target DNA interaction and mimicking the ST inhibitors such as DKAs.

Probing Integrase Inhibitor Binding in the Integrase Active Site—Separate integrase binding sites for donor (viral) and target DNA have been proposed, and inhibitors may bind differentially to these DNA-binding sites in order to inhibit selectively 3'-P and/or ST (1, 24). A 3'-P inhibitor (5CITEP, for example) may bind the donor DNA-binding site (24), whereas a
selective ST inhibitor (L-708906, for example) may bind the target DNA site at the interface of integrase, the two DNAs, and the two divalent metal ions (1). The U3(S) dA- and U2(S) dG-adducted DNA provided unique substrates to probe the binding of inhibitors to the integrase active site.

We observed position-specific effects of the BaP DE adducts on inhibition of integrase by three DKA-like compounds (Figs. 8 and 9 and summarized in Fig. 10). The U2 dG adduct caused an increase in inhibitor effectiveness against 3'-P. Hence, this adduct probably modifies the 3'-P active site to provide a better binding site for DKAs that are normally ST-selective, resulting in an increased inhibition of 3'-P (Fig. 10C). In contrast, the U3 dA adduct selectively enhanced 3'-P inhibition for L-870810 (Fig. 10F), suggesting that L-870810 interacts differently with the integrase 3'-P site compared with L-708906 and Ma-DKA.

The U3 dA adduct also affected ST inhibition. This adduct decreased the effectiveness of L-708906 and Ma-DKA but increased the effectiveness of L-870810 against ST (Fig. 10G). Presumably, the U3 dA adduct interferes with L-708906 and Ma-DKA isomers placed near the 3'-P site, whereas integrase tolerates bulky BaP DE adducts in the same region. The apparent contrast in the relatively small DNA backbone modifications that block catalysis with the large BaP DE adducts that permit catalysis suggests that integrase has specific backbone structure and flexibility requirements in the viral DNA substrate. Inhibition of phosphate inversion during nuclease cleavage may be a general nuclease resistance mechanism by nucleic acids containing backbone modifications. Oligonucleotides containing site-specifically placed BaP DE adducts provide unique tools for examination of inhibitor-binding sites at the integrase-DNA interface.

In summary, we found integrase inhibition by conformationally restricted nucleosides and anomeric isomers placed near the 3'-P site, whereas integrase tolerates bulky BaP DE adducts in the same region. The apparent contrast in the relatively small DNA backbone modifications that block catalysis with the large BaP DE adducts that permit catalysis suggests that integrase has specific backbone structure and flexibility requirements in the viral DNA substrate. Inhibition of phosphate inversion during nuclease cleavage may be a general nuclease resistance mechanism by nucleic acids containing backbone modifications. Oligonucleotides containing site-specifically placed BaP DE adducts provide unique tools for examination of inhibitor-binding sites at the integrase-DNA interface. BaP DE adduct interference has also proven useful with other DNA-interacting enzymes, including DNA topoisomerases I (47–51) and II (16) and DNA polymerases (34, 52–55).

Acknowledgments—We thank Dr. Kurt Kohn and Dr. Christophe Marchand for useful discussions and suggestions.
Integrase Accesses Supple DNA Backbones from the Minor Groove

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