Position-specific Suppression and Enhancement of HIV-1 Integrase Reactions by Minor Groove Benzo[a]pyrene Diol Epoxide Deoxyguanaine Adducts

IMPLICATIONS FOR MOLECULAR INTERACTIONS BETWEEN INTEGRASE AND DNA SUBSTRATES

The viral protein HIV-1 integrase is required for insertion of the viral genome into human chromosomes and for viral replication. Integration proceeds in two consecutive integrase-mediated reactions: 3'-processing and strand transfer. To investigate the DNA minor groove interactions of integrase relative to known sites of integrase action, we synthesized oligodeoxynucleotides containing single covalent adducts of known absolute configuration derived from trans-opening of benzo[a]pyrene 7,8-diol 9,10-epoxide by the exocyclic 2-amino group of deoxyguanaine at specific positions in a duplex sequence corresponding to the terminus of the viral US DNA. Because the orientations of the hydrocarbon in the minor groove are known from NMR solution structures of duplex oligonucleotides containing these deoxyguanaine adducts, a detailed analysis of the relationship between the position of minor groove ligands and integrase interactions is possible. Adducts placed in the DNA minor groove two or three nucleotides from the 3'-processing site inhibited both 3'-processing and strand transfer. Inosine substitution showed that the guanine 2-amino group is required for efficient 3'-processing at one of these positions and for efficient strand transfer at the other. Mapping of the integration sites on both strands of the DNA substrates indicated that the adducts both inhibit strand transfer specifically at the minor groove bound sites and enhance integration at sites up to six nucleotides away from the adducts. These experiments demonstrate the importance of position-specific minor groove contacts for both the integrase-mediated 3'-processing and strand transfer reactions.

HIV-1 integrase (integrase), along with reverse transcriptase and protease, is one of the three essential retroviral enzymes that can be targeted by antiviral compounds. Integrase catalyzes insertion of cDNA copies of the viral genome into human chromosomes. During this reaction, integrase multimers are associated with both ends of the viral cDNA and several host and viral proteins that together form preintegration complexes.

The biochemical reactions catalyzed by integrase can be studied in vitro with recombinant integrase and synthetic oligonucleotides. In vivo, integrase first catalyzes a cleavage reaction releasing a dinucleotide (5'-p-GT-3') from the 3'-end of each viral long terminal repeat (LTR) (3'-processing, 3'-P; Figs. 1A, 2A, and 2B). The exposed 3'-hydroxyl at the cleavage site is then used by integrase for nuclease attack upon a target DNA, resulting in insertion of the viral genome into a target chromosome (strand transfer, ST) (Fig. 2A). Cellular factors perform gap repair and ligation between the viral and host genome junctions. In vitro, a single DNA duplex consisting of the last 21 nucleotides of the HIV-1 U5 LTR (Fig. 1A) can be used to study both 3'-P and ST (Fig. 2A). The cleavage reaction (3'-P) is observed as accumulation of a 19-mer DNA band on a denaturing sequencing gel. Another identical duplex serves as the target DNA, and the ST reaction products (STPs) are observed as bands migrating higher than the substrate 21-mer in a denaturing sequencing gel (see Fig. 2C).

Elucidation of the molecular interactions between integrase and its DNA substrates (viral and chromosomal DNA) has proven challenging, and these interactions presently remain unknown. Models describing the arrangement of a simplified complex including integrase plus the viral and host DNA substrates have been proposed (1–4) based on biochemical and structural experiments. Biochemical studies have revealed specific contact points between several DNA bases and integrase amino acids. Integrase has an absolute requirement for the 5’-CA-3’ dinucleotide sequence immediately 5’ to the 3’-P site in the strand to be cleaved at the end of the viral LTR sequence (Fig. 1A, (5–8)). The efficiency of 3'-P is dramatically decreased by changes to the G immediately 5’ to the conserved CA dinucleotide (9, 10). The deoxyadenosine within the conserved CA has been shown to form a cross-link to Lys-159 of integrase (11). This Lys-159 residue also contacts the phosphate 5’ to the conserved deoxyadenosine in a two-domain structure of integrase (3). Mutagenesis showed that Tyr-143 and probably Gln-148 interact with the 5’-overhang (lower strand; Fig. 2A) resulting from upper strand 3’-P (11, 12). Disulfide cross-linking revealed proximity of the integrase amino acid 246 when mutated from glutamic acid to cysteine and the seventh base from the 5’-end of the lower strand of the U5 LTR (1). Although these four contacts have been useful for general alignment of
the viral DNA substrate relative to integrase, additional information is required for any higher resolution modeling or facilitation of structure-based drug design.

Ethylation interference has been used to locate phosphates that are critical for integrase catalysis (13). This method highlighted specific DNA backbone contacts required for ST near the insertion site. On the donor strand, the phosphate 5′ to the nucleophilic deoxyadenosine 3′-hydroxyl group, as well as the two phosphates on the complementary strand that are closest to the cleavage site, were important for ST. Within the target DNA, the two phosphates proximal to the ST site were important. Together, these observations suggest many stabilizing contacts between integrase, the tip of the cleaved LTR, and the site of ST.

We have used covalent adducts (Fig. 1B) derived from trans-opening of enantiomeric benzo[a]pyrene 7,8-diol 9,10-epoxides (BaP DE, isomer in which the epoxide oxygen and the benzylic 7-hydroxyl group are trans) by the exocyclic amino group of deoxyguanosine (dG) as probes of the interactions between DNA-processing enzymes, such as human and vaccinia topoisomerases (14–16), with the minor groove of DNA. These adducts in DNA exhibit well-defined structural motifs as shown by two-dimensional NMR studies in solution (17–19), with the hydrocarbon portion lying in the minor groove and oriented toward the 5′- or 3′-terminus of the adducted strand for the trans-(S) and trans-(R) adducts, respectively, where (S) and (R) refer to the absolute configuration at the point of attachment of the 2-amino group to the hydrocarbon (schematically illustrated in Fig. 1B). The trans-opened BaP DE dG adducts do not significantly disturb Watson-Crick base pairing or B-form DNA.

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Material and Methods

Oligonucleotide Synthesis—Unmodified and inosine-substituted oligonucleotides were commercially synthesized by IDT (Coralville, IA). Oligonucleotides containing the trans-opened BaP DE dG adducts were synthesized as described previously (20), using a manual coupling step for the added phosphoramidites. Oligonucleotides U17(R,S) and L4(R,S) (for notation, see Fig. 1A) were prepared from the mixed 10R/10S diastereomers of the BaP DE dG phosphoramidite (20) and were separated by reverse-phase high-performance liquid chromatography. Their absolute configurations were assigned from the long-wavelength CD spectra of the separated oligonucleotides, which exhibit positive bands in the 330–360 nm region for the 10R adduct and negative bands for the 10S adduct (21). All other adducted oligonucleotides were prepared from diastereomerically pure 10R or 10S phosphoramidites derived from the 10R and 10S diastereomers of the BaP DE dG adducts that had been separated as their O′-allyl di-2-tert-butyldimethylsilyl triacetates (22). Characterization of the diastereomerically pure phosphoramidites is given in the Supplemental Information. All adducted oligonucleotides were purified by high-performance liquid chromatography (see Supplemental Information for conditions and retention times).

Oligonucleotides were further purified on denaturing 20% polyacrylamide gels. Single-stranded oligonucleotides were 5′-labeled by T4 polynucleotide kinase (Amersham Biosciences, Piscataway, NJ) or 3′-labeled by terminal transferase (NEB, Beverly, MA) with cordycepin [α-32P]dATP (Perkin Elmer Lives Sciences) according to the manufacturers’ instructions. Unincorporated nucleotide was removed by mini Quickspin oligo column (Roche, Indianapolis, IN). 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 (23) 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 600 nM integrase, 20 nM duplex DNA, 7.5 mM MnCl2, 5 mM NaCl, 14 mM 2-mercaptoethanol, and 20 mM 4-morpholinepropanesulfonic acid, pH 7.2. This high concentration of integrase was used to maintain the multimer complex required for enzymatic activity. Results similar to those reported here with manganese were also observed with magnesium in the reaction mixture. 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 Molecular Dynamics 445 SI phosphorimager (Sunnyvale, CA). Densitometric analysis was performed using ImageQuant software from Molecular Dynamics.

The Schiff base cross-linking experiments were performed as described (24). Briefly, oligonucleotides containing a uracil at position U19 (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 facilitates ribose opening and formation of a Schiff base cross-link between an aldehyde group on the ribose and a nearby integrase lysine. The cross-links were stabilized by addition of 100 mM sodium borohydride. The cross-linked integrase-DNA products were separated from the substrate DNA by SDS-PAGE using 16% tricine gels (Invitrogen, Carlsbad, CA).

Results

We placed a single trans-opened BaP DE dG adduct at each of five positions within the terminal 21 bases of the HIV-1 U5 LTR outlined in Fig. 1A. The locations chosen for the BaP DE dG adducts are near the site of 3′-P and the prominent ST sites (see mapping of ST sites in Fig. 5). By convention, modified (dG adducted) oligonucleotides were named (U) and (L) when the adduct was attached on the upper or lower strand, respectively. The position of the adduct was counted from the 5′-end of the strand bearing the adduct. For instance, U17(S) refers to the modified duplex oligonucleotide with a BaP DE trans-(S) dG adduct at the 17th position relative to the 5′-end of the upper strand (see Fig. 1).

Binding of Integrase to Modified Oligonucleotides Containing BaP DE dG Adducts—Before investigating integrase catalytic reactions, we examined the binding of integrase to the adducted oligonucleotides using the Schiff base cross-link assay between integrase and its DNA substrate (24). None of the BaP DE dG adducts affected integrase binding to the modified DNAs (Fig. 1S, Supplemental Information).

Effect of Minor Groove BaP DE Adducts Close to the 3′-P Site—Two of the sites chosen to incorporate BaP DE dG adducts are located near the integrase 3′-P site: L4 and U17 (see Fig. 1A). The effects of trans-(R) and trans-(S) BaP DE dG adducts at the L4 position were evaluated on both the 3′-P and the ST reactions (Fig. 2). Integrase reactions are shown schematically in A, B, E, and F. The trans-(R) adduct (L4(R)) partially inhibi-
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Oligonucleotides used in the present study and structures and placement of the BaP DE dG adducts. A, sequence of the oligonucleotide corresponding to the terminal 21 bp of the HIV-1 LTR. Sites of individual adduct placement on the duplex are indicated by boxes. The positions of the adducts are counted from the 5' end (position 1) of each DNA strand with U and L, indicating dG adducts in the upper strand and lower strand, respectively. The black arrowhead shows the site of HIV-1 integrase-mediated 3'-P cleavage. B, chemical structure and differential orientation of the trans-(S) (S) and trans-(R) (R) adducts in the minor groove as shown for the U17(R) and (S) adducted oligonucleotides.

Fig. 2. Inhibition of both 3'-P and ST by BaP DE adducts at the L4 position near the 3'-P site. Asterisks indicate the position of the radioactive label. A, schematic diagram of in vitro integrase reactions (3'-P and ST) using full-length 5'-32P-labeled DNA. B, schematic representation of the L4(R) and L4(S) DNA adducts. The adducted dG is boxed. Orientation of the trans-(R) and trans-(S) adducts is shown between the two strands to indicate the binding orientation in the minor groove. The normal integrase 3'-P site (generating a 19-mer product) is indicated by a black arrowhead. A secondary integrase cleavage site (generating a 20-mer product) is indicated by a gray arrowhead. C, representative gel showing products of integrase reactions with DNA labeled at the 5' end of the upper strand (U5): lane 1, U5' labeled DNA alone; lanes 2–4, integrase (IN) reactions for the unmodified (U5'), L4(R), and L4(S) DNAs, respectively. D, the 19- and 20-mer cleavage products shown in C are quantified. E, schematic diagram of the in vitro integrase ST reaction using precleaved (3'-P) 5'-32P-labeled DNA. F, diagram of the precleaved L4(R) or L4(S) DNA duplexes. Conventions are similar to B. G, gel showing inhibition of STPs by the adducts with the precleaved DNAs. Labeling is the same as for C. Lane 1, DNA alone; lanes 2–4, integrase reactions with unmodified (U5'), L4(R), and L4(S) DNAs, respectively.
Fig. 3. Inhibition of 3'-P and ST by BaP DE dG adducts at the U17 position near the 3'-P site. A, schematic representation of the U17(R) and U17(S) adducted DNA substrates. For conventions used, see Fig. 2. B, representative gel showing the effects of the U17 adducts on the integrase (IN) reactions. Oligonucleotides were 5'-32P-end-labeled on the upper strand (see A). U5, unmodified DNA (lanes 1 and 4); (R) and (S), modified oligonucleotides containing the U17(R) (lanes 2 and 5) and U17(S) (lanes 3 and 6) BaP DE adducts. Lanes 1–3 show unreacted DNAs, and lanes 4–6 show integrase reactions. The dagger (†) indicates an oligonucleotide containing an adduct. C, diagram of 3'-P (precleaved) U17(S) adducted DNA substrate. D, representative gel showing the effects of the U17(S) BaP DE adduct on the integrase ST reactions with precleaved substrates. Lanes 1 and 3 show the 19-mer 5'-32P-end-labeled control and U17(S) DNAs, respectively. Lanes 2 and 4 show ST reactions (see Fig. 2E) with the unmodified and the U17(S)-adducted DNAs, respectively.

The effects of the U17(S) adduct on the ST reactions were examined directly using a precleaved substrate (see Fig. 2E) containing the trans-(S) BaP DE dG adduct at the U17 position to isolate the ST reaction. Using this adducted DNA (Fig. 3C), ST was not observed (Fig. 3D, lane 4).

Together, the data obtained with the L4 and U17 BaP DE adducted DNAs demonstrate that the presence of a BaP DE dG minor groove adduct two or three nucleotides 5' from the 3'-P site and spanning the minor groove adjacent to the 3'-P site interferes with both 3'-P and ST.

Role of the Guanine 2-Amino Group at the U17 and L4 Positions—Because the U17 and L4 BaP DE dG adducts were linked to the guanine 2-amino position in the minor groove, oligonucleotides containing inosine substitutions at the L4 (Fig. 4, A and C) and U17 (Fig. 4E) positions were examined to determine the role of the guanine 2-amino group on 3'-P and ST. The L4 inosine caused a marked decrease in 3'-P (81%) and ST (92%) (Fig. 4B, lane 3). ST was directly tested using a precleaved upper strand (Fig. 4C). The L4 inosine did not block ST (Fig. 4D, lane 3), indicating that it selectively inhibited 3'-P.

In the presence of the U17 inosine (Fig. 4E), 3'-P was similar to the control DNA, and ST was only partially reduced (by ~24%, Fig. 4F, lane 4). These results demonstrate the critical importance of the minor groove guanine 2-amino group at position L4 for 3'-P and its dispensability at an adjacent position in the upper strand (U17).

Mapping of the Integrase ST Sites in the U5 LTR Sequence—To examine the effects of BaP DE dG adducts at positions L5, L10, and U6 (see Fig. 1A) on ST reactions, we first had to map the ST sites within the U5 LTR. With the unmodified U5 LTR oligonucleotide labeled at the 5'-end of the upper strand, clusters of ST sites are consistently observed (see Figs. 2 and 3). However, the STP bands cannot be assigned unambiguously because they arise from both upper and lower strand STP (see Fig. 2A). To map the integration sites and therefore the effects of BaP DE adducts, we used 3'-end-labeling with cordycepin either on the upper (Fig. 5A) or the lower (Fig. 5B) strand. For consistency in referring to the 3'- and 5'-end-labeled products, we did not include the cordycepin in numbering the residues in the 3'-end-labeled oligonucleotides. Placing the radioactive label at the 3'-end of one of the strands of the DNA allows the mapping of strand-specific integration products. For example, only the STP on the left side of the diagram in Fig. 5A will be observed when 3'-labeled upper strand DNA is used (Fig. 5C). These STPs result from ST into the upper strand. The STP on the right side of Fig. 5A, resulting from ST into the lower strand, do not contain a radioactive label and therefore cannot be observed. Likewise, only the STPs illustrated on the right of Fig. 5B are detectable in the gel shown in Fig. 5D.

Various DNA size markers (M) matching several integration products were included so that the sizes of the STPs could be determined (Fig. 5, C and D). The upper 24, lower 24, and lower 25 markers match the sequence of the integration products and therefore have the same electrophoretic migration as the 24 and 25 STPs. The upper 30, upper 34, lower 30, and lower 32 markers differ in sequence from the predicted STPs and migrate slightly faster (by approximately half a base) than the corresponding STPs.

A map of preferred integration sites on unmodified DNA was created by analyzing the strand-specific pattern of integration bands (Fig. 5E). The intensity of a band on a sequencing gel corresponds to the frequency of integration events at a particular site. Densitometric analysis was used to create a graph illustrating band migration for each STP (Fig. 5E). The height of each peak was transferred into a bar-graph format and aligned with the substrate DNA sequence (Fig. 5E) to illustrate the locations of ST. On the upper strand, two clusters of STP peaks were observed: one around the U6 position generating STPs migrating between 31- and 35-mers, and the other near the 3'-P site generating STPs migrating between 22- and 24-mers. Several STPs migrating between the 22- and 24-mer STPs are indicated as a striped bar because these bands did not migrate at expected positions; therefore, the exact position of
Fig. 5. Mapping in vitro integration (ST) sites. A, schematic diagram of in vitro integrase reactions using 3'-32P-end-labeled DNA (asterisk) on the upper strand (U3'). The STPs observed are specific to upper strand integration. B, schematic diagram of in vitro integrase reactions using 3'-32P-labeled (asterisk) DNA on the lower strand (L3'). The STPs observed are specific to lower strand integration. C, representative gel illustrating STPs in the upper strand (U3'). Markers (M) are 3'-labeled with the sizes indicated (lane 3). D, representative gel illustrating STP in the lower strand (L3'). Markers are 3'-labeled with the sizes indicated (lane 6). The upper strand marker at position 24 and the lower strand markers at positions 24 and 25 correspond to the exact integration sequence. The upper strand markers at positions 30 and 34 and the lower strand markers at positions 30 and 32 have slightly different sequences and therefore do not migrate exactly as the corresponding integration products. E, densitometric analysis of STPs on both DNA strands. Each peak on the line graphs corresponds to a band on the gels shown in C and D. Column heights are derived from the height of the peaks shown for each strand. The striped column represents the peak of the darkest band migrating between the 22 and 24 STPs. The upper strand marker at position 23 was not observed. The dG positions at U6, L10, and L8, at which BaP DE adducts are to be placed, are boxed.

ST is an approximation. On the lower strand, two STP clusters were observed peaking around 24- and 32-mers, respectively. The 32-mer STP peak was adjacent to the L8 and L10 positions, where BaP DE dG adducts were located in the experiments to be described below. Strand-specific mapping was used for analyzing the effects of each adduct.

Effect of the L8, L10, and U6 BaP DE dG Adducts on ST—The L8 and L10 positions are near the prominent lower strand STP peak 32 (identified in Fig. 5). The data in Fig. 6, B and C, show the results of labeling of the 5'-end of the upper strand of the L8(R) and L8(S) adducted duplexes and provide a composite of the STP in the upper and lower strands. In contrast, 3'-end labeling of the upper strand (shown in Fig. 6, D and E) allows detection of upper strand STP only and may be compared with Fig. 5, C and E (U3'). None of the upper strand STPs from the L8(R) adducted oligonucleotides contain adducts, and thus they migrate at the same positions as the unmodified controls.

In the unadducted control (Fig. 6B, lane 2), the triplet of STPs with the most prominent peak at 32 (indicated by the double-headed arrow) is attributable to integration into the lower strand. Decreased intensity was observed for this 32 STP cluster with the L8(S) adduct, when compared with the control. Suppression of STPs at this site is presumably attributable to proximity of the adducts. Changes to the ST pattern resulting from integration into the intact upper strand induced by the BaP DE adducts at the L8 position are shown in Fig. 6, D and E. The L8(S) adduct slightly enhanced the upper strand 33, whereas the L8(R) adduct suppressed the 24 STPs and resulted in two new STPs at 23 and 25. The strong 23 STPs (Fig. 6D, lane 3) are not apparent in the composite products seen on 5'-end-labeling of the upper strand (Fig. 6B, lane 3) and may result from an integration that occurs only when the upper strand is extended by one base by the cordycepin label. The L8(S) adduct gave an anomalous, unidentified upper-strand integration product that migrated between the bands corresponding to 25 and 24 STPs.

We next investigated the effects of the BaP DE adducts at the L10 position (Fig. 7A), two nucleotides away from the L8 adducts described above. In the composite of upper and lower strand STPs (Fig. 7B, upper strand 5'-end-labeled), the 30 STPs are enhanced by the L10(S) adduct and completely suppressed by the L10(R) adduct. The 30 STP results from integration into the lower strand on the 5'-side of the adducted G and hence migrates normally because it does not contain an adduct. Integration into the lower strand on the 5'-side of the
L10 adducts would result in STPs with retarded migration (e.g., 32°) because of the presence of the adduct (Fig. 7, B and C). The L10(R), and to a lesser extent the L10(S), adduct inhibited formation of these 32° STPs. The pattern of STPs derived from integration into the upper strand near its 3°-end is quite similar for the L10 (Fig. 7, D and E) and L8 (Fig. 6, D and E) adducts. Similar to the L8(R) adduct (compare Fig. 6D), the L10(R) adduct enhanced the 23 and 25 STP, but in contrast to the L8(R) adduct, the L10(R) adduct did not significantly inhibit formation of the 24 STP. Similarly, the L10(S) adduct (Fig. 7D, lane 4), similar to the L8(S) adduct (Fig. 6D, lane 4), produced an anomalous band migrating slightly slower than the 24-mer marker.

The U6 position (Fig. 8) is in the center of a strong cluster of upper- and lower-strand STPs (see Fig. 5E). Analysis of STPs with this adducted substrate 5°-end labeled on the upper strand is complicated by the possible formation of STPs containing one or two BaP DE adducts (from lower- and upper-strand STPs, respectively). Consistently, integration products for both the U6(R) and U6(S) adducts (Fig. 8B, lanes 5 and 6) in the 32 cluster were retarded on electrophoresis (shifted up) relative to the unadducted STPs (Fig. 8B, lane 4). These bands may correspond to the same positions of integration, producing products with altered mobility attributable to the hydrocarbon. For both adducts the 31° STPs appeared to be enhanced at the expense of the 32° STPs. In addition, the U6 adducts decreased the amount of STPs corresponding to the cluster around the 24° STPs (arrows in Fig. 8, B and C). We could not map precisely the integration sites in the 24° cluster observed for the U6 adducted DNAs. Nevertheless, these data are consistent with inhibition of integration into the lower strand at sites 24 and 25 opposite to the position of adduct attachment (Fig. 8A).
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**Fig. 8.** Effect of BaP DE dG adducts at the U6 position on ST reactions. For conventions and notation, used see Fig. 6. A, positions of 3'-P and ST sites and orientations of the adducts. B, integrate reactions with 5'-labeled upper strand DNA substrates (U5') (see Fig. 3A). 3'-P, resulting in a 19-mer, and ST are indicated on the left. Lane 1, unmodified DNA alone (U5'). Lanes 2 and 3, added U6(R) and U6(S), respectively. Lanes 4–6, integrate reactions with unadded (U5'), U6(R), and U6(S) DNAs, respectively. Daggers (right side of the gel) indicate products of 3'-P (19') and STPs containing an adduct. C, densitometric analysis comparing STPs for the unadducted DNA (top), U6(R) (middle), and U6(S) (bottom).

**DISCUSSION**

Chiral BaP DE adducts were incorporated into five different positions of individual oligonucleotides derived from the HIV-1 U5 LTR to explore interactions between HIV-1 integrase and the minor groove of substrate DNA. Because noncovalent binding of DNA to HIV-1 integrase was unaffected by the presence of the adducts, they could be used to study minor groove contacts between integrase and DNA. We observed position-dependent effects of the adducts on both 3'-P and ST. Specifically, 1) 3'-P and ST were blocked by adducts near the 3'-P site; 2) ST was blocked at the position of adduct minor groove occupancy; and 3) some ST sites were enhanced by adducts positioned remotely from those sites.

**Importance of the L4 Minor Groove Contacts for 3'-P—Stereo-specific inhibition of integrase 3'-P on the upper strand was observed for the lower strand L4(R) and L4(S) adducts that lie in the minor groove adjacent to the conserved 5'-CA-3' nucleotides, which are required for integrase activity (5–8). Furthermore, we found that the L4 guanine 2-amino group is also critical for efficient 3'-P because inosine substitution blocked integrase 3'-P.

Orientation of the L4 adducts proximal to the 3'-P site (*) is illustrated in Fig. 9. These space-filling illustrations were derived from two-dimensional NMR structures of adducted duplex DNAs with a different sequence from the present integrase substrate (17–19). They serve only to show the location of the scissile phosphate in relation to the BaP DE adducts for discussion purposes. Inhibitory effects of the L4 adducts on 3'-P were in agreement with the directionality of adduct extension in the minor groove relative to the cleavage site. Specifically, 3'-P was selectively blocked by the L4(S) adduct, which directly overlaps the minor groove at the 3'-P site (Figs. 1A and 9). This is in contrast to the L4(R) adduct, which extends away from the 3'-P site and allows some 3'-P. This defines the importance of the minor groove region that is covered by the L4(S) adduct for 3'-P. Stereospecific inhibition by trans-(R) and trans-(S) BaP DE adducts was also observed for DNA cleavage by vaccinia and human topoisomerases (15, 16). Both of these type IB topoisomerases perform site-specific cleavage and religation of DNA via a DNA-(3'-phosphotyrosyl)-enzyme intermediate. For both topoisomerases, the non-interfering diastereomer extended away from the scissile phosphate, whereas the inhibitory diastereomer extended toward the cleavage site. These results imply the importance of minor groove or guanine 2-amino interactions between the enzymes and DNA and show that BaP DE adducts are useful tools for examining enzymatic mechanisms.

The L4 guanine 2-amino group appears to be an important contact for efficient 3'-P, because decreased cleavage was observed with both the adducted and inosine-substituted DNA substrates. We observed similar binding of HIV-1 integrase to the control DNA and both of the L4 adducted DNAs (Fig. 15), implying that the L4 free amino group is not required for binding by HIV-1 integrase. In contrast, modified bases showed that the absence of the L4 guanine 2-amino group resulted in a loss of DNA binding and 3'-P by human T-cell leukemia virus type II integrase (25). It was hypothesized that human T-cell leukemia virus type II integrase may initiate binding of the viral DNA end through a minor groove interaction but may use required major groove contacts for integrase catalysis (25).

Unlike human T-cell leukemia virus type II 2, HIV-1 integrase may exhibit different binding requirements because DNA binding was unaffected by any of the minor groove BaP DE adducts. However, 3'-P was almost completely inhibited by the L4(S) adduct (and both U17(R) and (S) adducts, see below) in the minor groove, as well as the L4 inosine substitution.

**Role of Minor Groove Contacts Adjacent to U17 for 3'-P**—The U17 guanine, 2 bp removed from the 3'-P site, is critical for activity by HIV-1 integrase. An abasic site at this position prevents 3'-P (10). In contrast, we found that the U17 guanine 2-amino group is not required for 3'-P because substitution for this base by inosine (lacking the 2-amino group) did not affect 3'-P. Moreover, inosine substitution at U17 decreased ST by only 24%, implying that the 2-amino group is not required for the ST reaction. Therefore, the inhibition of 3'-P and ST by U17(R) and U17(S) adducts may be ascribed to the presence of the minor groove adducts rather than to a direct involvement of the U17 guanine 2-amino group.

The relative orientations of the adduct and the phosphodiester bond that is cleaved in 3'-P are illustrated in Fig. 9 for R and S adducts at U17 as well as at L4. Interestingly, both U17(R) and U17(S) adducts inhibit 3'-P, although U17(S) does not directly overlap the scissile phosphodiester bond (*). Thus, contacts distal to this cleaved bond must be critical for 3'-P. The only adduct of these four that permits significant 3'-P is L4(R), which occupies approximately the same linear space in the minor groove as the inhibitory U17(R) (Fig. 9). Therefore, L4(R) and U17(R) are not equivalent for integrase interaction. Integrase might be more inhibited when the adduct is covalently linked to the scissile strand (U17 position) than the non-scissile strand (L4 position), as was suggested recently for DNA topoisomerase II (26). Also, because they are on opposite strands, the U17(R) and L4(R) adducts have opposite orientations relative to the site of 3'-P. Specific steric or hydrogen bonding interactions mediated by the bulker and more exposed trihydroxy tetrahydrobenzo-ring (relative to the pyrene moiety) may play a role in the differential effects of these two adducts. For example, if minor groove interference is inhibitory in a region one or two base pairs 5' to the 3'-P cleavage site (to the lower left in Fig. 9), then this bulker portion of the adduct may present less of a block to the enzyme when closer to the 3'-P site as in L4(R) as compared with L17(R). Interestingly, an opposite
effect of BaP DE dG adduct orientation was observed for DNA cleavage by vaccinia topoisomerase, where greater inhibition of cleavage was observed when the bulky trihydroxy tetrahydrobenzo-ring was oriented toward the cleavage site (16).

Global Versus Site-specific Inhibition of ST by the BaP DE Minor Groove Adducts—Unlike 3'-P, ST was globally inhibited by BaP DE adducts positioned adjacent to the DNA 3'-hydroxyl group, which acts as the nucleophile for the ST reactions. Binding of the substrate for the cleavage reaction was not perturbed by the presence of the BaP DE-dG adducts, as demonstrated by Schif base cross-linking experiments (see Fig. 1S, Supplemental Material). However, inhibition of ST suggests that these bulky adducts prevent the proper binding of this target DNA to integrate and/or the catalysis of the ST reaction. ST was also inhibited at and near adducts at the L8, L10, and possibly the U6 positions. These adducts could inhibit ST in three ways: 1) by blocking appropriate positioning of the nucleophilic 3'-hydroxyl of the donor DNA; 2) by blocking a required protein-minor groove contact; or 3) by blocking target DNA binding. In contrast to the site-specific 3'-P cleavage, selection of an ST site occurs in an apparently sequence-independent manner. Analysis of the crystal structures of 60 protein-DNA complexes to determine minor groove components important for recognition of DNA by drugs and proteins (27) showed that purine N3, pyrimidine O2, deoxyguanine N2, and deoxyribose O4 were the only minor groove groups involved in interactions between DNA and proteins, drugs, and water. Additionally, sequence-independent hydrogen-bond acceptors in the minor groove lie in identical positions for all four Watson-Crick base pairs (28). Therefore, it is likely that the BaP DE adducts may block integrase side-chain interaction with minor groove hydrogen-bonding groups required to position the acceptor DNA in a sequence-independent manner, prohibiting ST at the site of adduction. For example, the deoxyguanosine N2 amino group forms many water-mediated contacts in protein/DNA complexes (27) that may be blocked by BaP DE dG adducts.

Enhancement of ST at Sites Flanking BaP DE Adducts—ST was enhanced at several insertion sites remote from the BaP DE dG adducts at the L8, L10, and U6 positions, notably to give the 23 and 25 STPs with L8(R) and L10(R) adducts and the 31 STPs with U6(S) and (R) adducts. This enhanced ST may be a result of a shift of the total ST activity of integrase to new sites. The low sequence specificity of integrase target site selection may promote integration at less preferred ST sites when preferred sites are blocked by the BaP DE adducts or by structural perturbations in their immediate vicinity. NMR structures of BaP DE dG adducted DNA have shown that the hydrocarbon occupies the minor groove, which is deeper in the groove.

B-form helix conformation with normal base pairing (17–19). However, recent molecular dynamics calculations on these structures, based on the NMR data, have indicated that the minor groove is significantly (2–4 Å) widened at the site of attachment of these dG adducts and 1–2 bp away, particularly for the S adduct (29). A similar effect on human topoisomerase I specificity was observed with BaP DE dG adducts at or near a normal cleavage site, which resulted in suppression of DNA cleavage at the adduct site and enhancement of cleavage at distal sites (14, 15).

Inhibition of HIV-1 by Targeting the LTR Minor Groove—Integrase is an important clinical target for the inhibition of HIV replication. Synthetic polyamines such as the lexitropsins selectively bind the minor groove of the conserved AAGAAT stretch of the HIV-1 LTR and inhibit integrase at nanomolar concentrations (30). The results presented here suggest that targeting the minor groove of the HIV-1 DNA near the integrase 3'-P site may also effectively inhibit integrase activity. Polyamines specifically binding the A+T-rich tip of the Moloney murine leukemia virus LTR exhibit nanomolar inhibition of integrase and subnanomolar affinity for the Moloney murine leukemia virus LTR (31), supporting the viability of this approach for HIV. Both the HIV-1 U3 and U5 LTRs terminate in sequences that are site specifically bound by integrase (5'-GCAGT-3' for the U5 LTR and 5'-CCAGT-3' for the U3 LTR). Additionally, simultaneously targeting of integrase and the DNA minor groove with the same inhibitor may increase inhibitor specificity. Minor groove-binding inhibitors recognizing this sequence, or other types of inhibitors that specifically prevent interaction between integrase and the minor groove of the LTR tip, could interfere with provirus integration and therefore with viral replication.

Acknowledgment—We thank Dr. Suse Broyde (New York University) for providing us with the coordinate files for the DNA containing trans-opened BaP DE dG adduct.

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