Chemical Trapping of Ternary Complexes of Human Immunodeficiency Virus Type 1 Integrase, Divalent Metal, and DNA Substrates Containing an Abasic Site

IMPLICATIONS FOR THE ROLE OF LYSINE 136 IN DNA BINDING*

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We report a novel assay for monitoring the DNA binding of human immunodeficiency virus type 1 (HIV-1) integrase and the effect of cofactors and inhibitors. The assay uses depurinated oligonucleotides that can form a Schiff base between the aldehydic abasic site and a nearby enzyme lysine ε-amino group which can subsequently be trapped by reduction with sodium borohydride. Chemically depurinated duplex substrates representing the 5′ end of the HIV-1 DNA were initially used. We next substituted an enzymatically generated abasic site for each of 10 nucleotides normally present in a 21-mer duplex oligonucleotide representing the 5′ end of the HIV-1 DNA. Using HIV-1, HIV-2, or simian immunodeficiency virus integrases, the amount of covalent enzyme-DNA complex trapped decreased as the abasic site was moved away from the conserved CA dinucleotide. The enzyme-DNA complexes formed in the presence of manganese were not reversed by subsequent addition of EDTA, indicating that the divalent metal required for integrase catalysis is tightly bound in a ternary enzyme-metal-DNA complex. Both the N- and C-terminal domains of integrase contributed to efficient DNA binding, and mutation of Lys-136 significantly reduced Schiff base formation, implicating this residue in viral DNA binding.

Efficient replication of retroviral DNA requires establishment of the proviral state, i.e. the integration of a DNA copy of the viral genome, synthesized by reverse transcriptase, into a chromosome of the host cell. Integration is catalyzed by the viral integrase protein. Prior to integration, two nucleotides are excised from each 3′ end of the linear, blunt-ended, viral DNA. This 3′-processing reaction exposes the 3′-hydroxyl of a CA dinucleotide which is conserved among all retroviruses. Each of these 3′-hydroxyl ends of the viral DNA are then joined to chromosomal DNA in the subsequent DNA strand transfer step. DNA strand transfer is an isosequential transesterification reaction. HIV-1 integrase catalyzes a nucleophilic attack of these 3′-hydroxyl group at the processed viral ends on a pair of phosphodiester bonds staggered by five base pairs in the target DNA. Completion of the integration process requires removal of the two unpaired nucleotides at the 5′ ends of the viral DNA and gap repair reactions that are thought to be accomplished by cellular enzymes. (See Katz and Skalka (1) and Vink and Plasterk (2) for recent reviews on retroviral DNA integration.)

Retroviral integrases have been shown to bind DNA nonspecifically (3–6), and this activity has been localized to the C terminus of integrase by Southwestern blotting (7–9), nitrocellulose filter binding (10), and UV cross-linking (9, 11). In the present report, we describe a novel assay for the DNA binding activity of HIV-11 integrase using modified DNA oligonucleotides containing amino-reactive abasic sites and sodium borohydride stabilization. We then determined the effects of divalent metal ions, site-directed and deletion mutagenesis, and inhibitors on the formation of integrase-DNA complexes. We provide evidence for a tight association of the manganese with the enzyme-DNA complex such that this complex was not reversible upon addition of EDTA. We also examined the role of lysine 136 in DNA interactions both proximal and distal to the conserved CA dinucleotide.

MATERIALS AND METHODS

Preparation of Radiolabeled DNA Substrates—The following oligonucleotides were high performance liquid chromatography purified by and purchased from Midland Certified Reagent Company (Midland, TX): AE117, 5′-ACTCTAGAGATTTCCTCCACAC-3′ and deoxyuridine analogs (see Fig. 3A); AE118, 5′-GTGTTGGAAAATCTCTAGACTG-3′ and deoxyuridine analogs (see Fig. 3A); AE118S, 5′-CTCTAGGAAATCTCTAGCTG-3′ and deoxyuridine analogs (see Fig. 3A); RM22M, 5′-T-ACGCTTAGAGATTTCCTCCACAC-3′; RM35A, 5′-GACCCCTTTTATGCATGTTGGAAAATCTCTAGCTG-3′; RM35B, 5′-ACGCTTAGAGATTTCCTCCACAC-3′; RM30, 5′-GTGTTGGAAAATCTCTAGCTG-3′; RM10, 5′-A-CACGCTAGAGATTTCCTCCACAC-3′; RM32, 5′-ACGCTGAGATTTCCTCCACAC-3′; RM33, 5′-AACGGGAGTTCCTCCACAC-3′; RM50B, 5′- ACGCTGAGATTTCCTCCACAC-3′; RM50A, 5′-ACTCTAGAGATTTCCTCCACAC-3′; RM100, 5′-A-CACGCTAGAGATTTCCTCCACAC-3′; RM105, 5′-ACGCTGAGATTTCCTCCACAC-3′; RM110, 5′-ACGCTGAGATTTCCTCCACAC-3′; and RM115, 5′-ACGCTGAGATTTCCTCCACAC-3′.

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The abbreviations used are: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; LTR, long terminal repeat; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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To analyze the extent of strand transfer using the "precleaved" substrate AE118S and deoxyuridine analogs (see Fig. 3A), the 5'-end-labeled, annealed to AE117, and column-purified as above.

To analyze the choice of nucleophile for the 3'-processing reaction, AE118 and deoxyuridine analogs (see Fig. 3A) were 3'-end-labeled using NaOH for 30 min at 30°C. The extent of cleavage of the oligonucleotide at a final concentration of 1 μM of the radiolabeled AP site-containing DNA with 166 mM sodium borohydride was determined to be 100%, implying that all of the uracil from the substrate had been excised.

Chemical and Enzymatic Depurination—Duplex oligonucleotides were depurinated by incubation of 35 μl of end-labeled duplex (500 nM stock concentration) with an equal volume of formic acid (50% final concentration) for 20 min at 30°C. The reaction was then dried under vacuum and redisolved into 150 μl of water, dried under vacuum again, and redisolved in 35 μl of water.

Duplex oligonucleotide substrates containing a single enzymatically generated abasic site were created as follows. Analogs of AE118 (see sequence above and in Fig. 3A) were synthesized so that one deoxyuridine replaced each of the wild-type nucleotides in this strand. For example, substrates 1 and 11 (see Fig. 3A) had the sequences 5'-GTGTGGAAAAATCTCTAGCUGT-3' and 5'-GTGTGGAAUATCTCTAGCAGT-3', respectively. Each of these single strands was then radiolabeled and annealed to the complementary strand AE117 as described above. The uracil was removed from duplex oligonucleotides containing deoxyuridine by incubation of 40 μl of end-labeled duplex (500 nM stock concentration) with 1 unit of uracil DNA glycosylase (Life Technologies, Inc.) at 30°C. The reaction was then loaded on a G-25 Sephadex spin column as before.

The extent of AP site formation was determined by incubation of 0.5 μM of the radiolabeled AP site-containing DNA with 166 mM sodium hydroxide for 30 min at 30°C. The extent of cleavage of the oligonucleotide by β- and δ-elimination reactions was quantitated and determined to be 100%, implying that all of the uracil from the substrate had been excised.

Integrase Proteins—Recombinant HIV-1 integrase was purified as described elsewhere (13). Purified recombinant wild-type SIV integrase was a generous gift of Drs. R. Craigie and A. Hickman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. A plasmid encoding purified recombinant wild-type SIV integrase was a generous gift of Drs. R. Craigie and A. Hickman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. A plasmid encoding purified recombinant wild-type SIV integrase was a generous gift of Drs. R. Craigie and A. Hickman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. A plasmid encoding purified recombinant wild-type SIV integrase was a generous gift of Drs. R. Craigie and A. Hickman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD.

3'-Processing and Strand Transfer Assays—Integrase at a final concentration of 200 (for HIV-1 and HIV-2) or 600 nM (for SIV) was mixed with 20 nM of the 5'-end-labeled linear oligonucleotide substrate in reaction buffer (50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (v/w), 7.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, and 25 mM MOPS, pH 7.2). When used, deletion mutants were used at a concentration of 25 mM unless otherwise specified. Reactions were then heated at 95°C, allowed to cool slowly to room temperature, and run on a 6-25% gel as described previously (14).

Electrophoresis and Quantitation—Reactions were quenched by the addition of an equal volume (16 μl) of Maxam-Gilbert loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 mM Tris borate pH 8.3, 2 mM EDTA, 20% acrylamide, 8 μM urea). Gels were dried, exposed in a Molecular Dynamics PhosphorImager cassette, and analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Schiff Base Formation and Chemical Trapping—A solution of sodium borohydride (1 mM stock concentration, unless otherwise specified) was prepared fresh prior to the start of the experiment. Integrase was incubated with the oligonucleotide containing the chemical or enzymatically generated abasic site in reaction buffer (as described in "3'-Processing and Strand Transfer Assays") for 2 min at room temperature (unless otherwise specified). Sodium borohydride (0.2 M) was then added (0.1 x final concentration, unless otherwise specified), and the reaction was continued for an additional 5 min. An equal volume (16 μl) of 2 × SDS-PAGE buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added to each reaction, and the reaction was heated at 95°C for 3 min prior to loading a 20-μl aliquot onto a 12% SDS-polyacrylamide gel. The gel was run at 120 V for 1.5 h, dried, and exposed on a PhosphorImager cassette. For inhibition of DNA-binding experiments, integrase (200 nM) was preincubated with the inhibitor (at the indicated concentration) for 30 min at 30°C prior to the subsequent addition of the radiolabeled viral DNA substrate (20 μM) and borohydride. Gels were analyzed using a Molecular Dynamics PhosphorImager.

UV Cross-linking Experiments—The method used has been described by Yoshinaga et al. (15). Briefly, integrase was incubated with substrate in reaction buffer as above for 5 min at 30°C. Reactions were then irradiated with a UV transilluminator (254-nm wavelength) from 3 cm above (2.4 milliwatts/cm²) at room temperature for 10 min. An equal volume (16 μl) of 2 × SDS-PAGE buffer was added, and an aliquot was electrophoresed as described above.

RESULTS

Trapping a Schiff Base Formed between HIV-1 Integrase and a Chemically Depurinated Duplex U5 LTR Substrate—The acid-catalyzed depurination of DNA results in the formation of abasic sites (16) which are predominantly a mixture of the α- and β-anomers of the hemiacetal (17) (Fig. 1). These anomers exist in equilibrium with the acyclic aldehydic form, which accounts for approximately 1% of abasic sites (18). Because abasic sites do not significantly perturb the structure of B-DNA (19, 20), we reasoned that introduction of one such lesion into a duplex U5 LTR oligonucleotide may not alter its ability to act as a substrate for HIV-1 integrase. Furthermore, HIV-1 integrase has previously been shown to form a Schiff base (an imine linkage) between oxidized ATP and, presumably, a lysine ε-amino group in the proximity of this nucleotide binding site (21). We therefore tested the ability of several U5 LTR duplexes of increasing length to form a Schiff base upon integrase binding (Fig. 1). The Schiff base could be detected after reduction of the DNA-enzyme complex with sodium borohydride to stabilize the otherwise labile imine (Fig. 1). The resulting covalent enzyme-DNA complex was detected by SDS-PAGE analysis and PhosphorImager visualization.

The sequences of the duplexes used are shown in Fig. 2A. As seen in Fig. 2B, duplex substrates which were not depurinated (both panels, lanes 2) or which were depurinated but not subjected to borohydride reduction (both panels, lanes 5 and 8) could not form a detectable DNA-enzyme complex. Depurinated substrates generated a DNA-enzyme complex which was detected after borohydride reduction (both panels, lanes 6 and 9). The ability to form a Schiff base with the abasic site was observed whether the top or bottom strand was depurinated (both panels, compare lanes 6 and 9). If the abasic abasic site was reduced to the primary alcohol (with borohydride) prior to the addition of integrase, a DNA-enzyme complex could not be detected (both panels, lanes 10 and 11), as would be
HIV-1 Integrase-Viral DNA Complex

Fig. 2. Formation of a Schiff base between HIV-1 integrase and chemically depurinated duplex U5 oligonucleotide substrates of increasing length. A, sequence of the oligonucleotide substrates. B, PhosphorImager pictures from a typical experiment using the 21- and 35-mer substrates. 'U5' and 'U5p' represent the normal (nondepurinated) DNA and the depurinated DNA, respectively. Lane 4 corresponds to depurinated DNA 5' end labeled on the top strand, and 'U5p' corresponds to depurinated DNA 5' end labeled on the top strand which has been reduced by boehorodrde to the alcohol before integrase addition. Lanes 1, 4, 7, and 10, DNA alone; lanes 2, 5, 8, and 11, DNA plus magnesium; lanes 3, 6, and 9, DNA plus integrase and boehorodrde. Lane 12 shows the results of UV cross-linking of the nondepurinated DNA with integrase. Molecular mass markers (in kDa) are shown to the right of the gels. C, metal ion requirement and specificity for Schiff base formation with chemically depurinated U5 oligonucleotide substrates (see A) labeled on the top strand. Molecular mass markers (in kDa) are shown to the right of the figure. The left, middle, and right panels are PhosphorImager pictures from a typical experiment using the 21-, 35-, and 50-mer strands, respectively. Lanes 1, DNA alone; lanes 2 and 3, in the presence of 2 mM EDTA without (lane 2) or with (lane 3) boehorodrde addition; lanes 4 and 5, in the presence of 2 mM MnCl$_2$ without (lane 4) or with (lane 5) boehorodrde addition; lanes 6 and 7, in the presence of 2 mM MgCl$_2$ without (lane 6) or with (lane 7) boehorodrde addition.

expected from the loss of an electrophilic carbonyl. With any of the three substrates, the DNA-enzyme complex demonstrated the expected mobility of a complex of the DNA and an enzyme monomer (39 and 43 kDa for the left and right panels, respectively) and had a similar mobility as a DNA-enzyme complex generated by UV cross-linking (11, 15) (both panels, lanes 12). A DNA-enzyme complex was also obtained when a 50-mer depurinated duplex oligonucleotide substrate was used (data not shown). After treatment with boehorodrde, a very low level of DNA-enzyme complex was detectable with control substrates which were not depurinated (both panels, lanes 3), presumably due to a low level of spontaneous depurination in DNA (16).

Recent photocross-linking studies have shown that binding of full-length wild-type HIV-1 integrase does not require a metal ion (11, 15). Furthermore, both 3'- and strand transfer can be efficiently performed by HIV-1 integrase using either manganese or magnesium (22, 23). We therefore determined the extent of Schiff base formation using substrates of increasing length in the absence or presence of divalent metal ion. As seen in Fig. 2C, using the 21-mer substrate, the substitution of EDTA for a divalent metal ion reduced the formation of enzyme-DNA complexes (left panel, lane 3). The presence of either manganese (left panel, lane 5) or magnesium (left panel, lane 7) enhanced the amount of Schiff base formed. Manganese induced a higher level of DNA-enzyme complex than magnesium when the 21-mer duplex was used (left panel, compare lanes 5 and 7). Similarly, a DNA-enzyme complex was also observed with either the 35- or 50-mer duplex oligonucleotide substrates (middle and right panels). Interestingly, the extent of DNA-enzyme complex formation was less dependent on the presence of divalent metal ion when the longer 35- or 50-mer duplexes were used (all panels, compare lanes 3 and 5). We conclude that, with the 21-mer substrate, the enhanced level of DNA binding by integrase in the presence of manganese versus magnesium parallels the enhanced catalytic activity observed in the presence of manganese. However, as the length of the substrate is increased, the ability of integrase to efficiently perform its 3'-processing activity in magnesium is also increased (24), consistent with results from our DNA binding assay (Fig. 2C).
actually increased in all cases (at −5 or −7). This decrease was observed whether the blunt-ended or the "precleaved" duplex substrates were used (Fig. 3B, compare lanes 1 through 19 and 20 through 25).

A kinetic study of Schiff base formation showed that a time-dependent increase in the extent of Schiff base formation was evident using each of the oligonucleotide substrates. Although substrates −5 through −11 displayed only a small increase in complex formation over time, substrates −2 and −5, exhibited significant increases in the amount of complex formation (data not shown).

In our efforts to optimize the binding conditions, we also investigated the effect of varying the substrate DNA concentration. We found that the trend observed in Fig. 3 was reproducible over a DNA oligonucleotide concentration range of 0.2 to 75 nM (data not shown).

Detection of Metal Ion-Integrase Complexes—The metal ion specificity for Schiff base formation with each of the abasic site-containing oligonucleotides was also tested in an attempt to probe enzyme interactions both close to and further away from the conserved CA dinucleotide. Recently, the crystal structure of ASV integrase complexed with a single manganese ion has been solved (27). This ion is coordinated to the carboxylate groups of the active site aspartates of integrase and uses water to complete its octahedral coordination. Possibilities still exist for the DNA substrate to complete the coordination sphere of the metal or for the DNA substrate to bring a second metal into the active site.

As seen in Fig. 4A, a low level of DNA-enzyme complex was detected with all the substrates tested in the presence of EDTA, but the amount of complex formed was strongly enhanced by the presence of divalent cation, consistent with results obtained from the chemically depurinated oligonucleotide substrates (see Fig. 2C). A difference was observed, however, in that the metal ion specificity for Schiff base formation did not differ significantly depending on the location of the abasic site, with manganese and magnesium generating comparable levels of Schiff base with all substrates tested (Fig. 4, A and B, compare conditions C and D and Fig. 2C, left panel, compare lanes 5 and 7).

The role of the metal ion in integrase structure and function has recently been investigated. Besides its role in catalysis, the
metal ion (either manganese or magnesium) is required to promote stable complex formation (28–30), possibly by promoting specific interactions between integrase protomers, leading to protein multimerization (30, 31). We tested the stability of the metal ion-promoted DNA binding activity of HIV-1 integrase by adding EDTA prior to (Fig. 4, C and D) or after (Fig. 4, E and F) the addition of DNA. As seen in Fig. 4C, an integrase-DNA complex was detected in the presence of either EDTA (lane 2) or manganese (lane 8), although preincubation of the enzyme in manganese greatly enhanced the level of DNA binding, consistent with data from Fig. 4, A and B. Addition of increasing concentrations of manganese after preincubation of integrase in 2 mM EDTA resulted in only a slight increase in DNA binding (Fig. 4, C, lanes 3–7, and D). Increasing concentrations of EDTA after preincubation of integrase in 2 mM MnCl$_2$ resulted in an EDTA concentration-dependent reduction in the level of DNA binding (Fig. 4, C, lanes 9–13, and D).

However, when each of these additions was performed after the addition of DNA (i.e., after a ternary integrase-metal-DNA complex had formed), different results were obtained (Fig. 4, E and F). For example, addition of increasing concentrations of manganese after preincubation of integrase in 2 mM EDTA resulted in approximately 10-fold increases in the DNA binding at concentrations equimolar to or in excess of the EDTA concentration (Fig. 4, E, lanes 5 and 6, and F). Addition of EDTA after preincubation of integrase in 2 mM MnCl$_2$ did not result in a significant reduction in the level of DNA binding (Fig. 4, E, lanes 9–13, and F). We conclude that preformed binary integrase-manganese complexes can be dissociated by EDTA, resulting in a reduction in the level of DNA binding by the enzyme, but that preformed ternary integrase-manganese-DNA complexes are stable in the presence of EDTA.

**Binding of HIV-1 Integrase to Different DNA Substrates**—HIV-1 integrase can bind both HIV-1 LTR and non-LTR DNA (3–6), consistent with its reaction mechanism, in which a viral DNA strand is inserted into a target DNA strand and with genetic data demonstrating lack of strict target site selectivity in the integration reaction (32, 33).

The level of Schiff base formation with substrates −2 and −11 (see Fig. 3A) was compared to that obtained with either a mutated form of −11 (−11$_{GCA}^{HET}$) or an heterologous oligonucleotide in which an abasic site was generated at an analogous location as in the −2 oligonucleotide (−2$_{HET}$). It has previously been demonstrated that mutation of the GCA trinucleotide to a CGT (as in −11$_{GCA}^{HET}$) reduces 3′-processing activity to about 7% (34). The same study showed that the heterologous oligonucleotide substrate (denoted −2$_{HET}$) was not processed by integrase. Integrase was able to form a Schiff base with all of these substrates (data not shown). We also found that HIV-1 integrase was able to form a Schiff base with single strands (where only the processed strand was present) of the substrate shown in Fig. 3A, consistent with previous results from UV cross-linking (11). We conclude that, in a 21-mer duplex substrate (e.g., those in Fig. 3A), integrase does not demonstrate highly selective binding to one sequence over others, consistent with binding of integrase to a variety of chromosomal sequences (32).

We also tested the ability of HIV-1 integrase to form a Schiff base with a shorter (16-mer) version of the 21-mer −2 duplex (Fig. 5A). This substrate has previously been shown to support both the 3′-processing and strand transfer activities of HIV-1 integrase (34) (see Fig. 5D). The level of DNA binding with this
substrate was a hairpin 16-mer duplex oligonucleotide representing the U5 end of the LTR with a two base (TT) loop proximal to the processing site. Lanes 1–3, substrate was a hairpin 16-mer duplex oligonucleotide representing the U5 end of the LTR; lanes 4–6, substrate was a hairpin 16-mer duplex oligonucleotide representing the U5 end of the LTR with a two base (TT) loop distal to the processing site; lanes 7–9, substrate was a hairpin 16-mer duplex oligonucleotide representing the U5 end of the LTR; lanes 10–14, DNA alone; lanes 15–21, with integrase at a final concentration of 5, 10, 20, 40, 80, and 160 nM, respectively.

**Schiff Base Formation with Site-directed and Deletion Mutants of HIV-1 Integrase**—UV cross-linking studies have shown that residues 213–266 of the C terminus of HIV-1 integrase are required for efficient cross-linking to the linear duplex U5 oligonucleotide in the absence of metal ion (11). In fact, a deletion mutant containing only residues 215–270 can bind to DNA in the presence of 2 mM EDTA (9). However, IN1–212 can bind to the linear duplex in the presence of MnCl₂ at 2.56 μM protein concentration (11). We analyzed the binding of site-directed and deletion mutants of HIV-1 integrase to three DNA duplex oligonucleotides containing an abasic site (see Fig. 3A). As seen in Fig. 6A, HIV-1 integrase containing the F185K/C280S mutations, which make the protein more soluble without compromising catalytic activity (19), was able to bind to the DNA substrates (lanes 3, 10, and 17) as well as wild-type integrase (lanes 2, 9, and 16). Elimination of the zinc finger, either by site-directed mutagenesis of the two histidines in this domain (lanes 4, 11, and 18) or by deletion of the N terminus (lanes 6, 13, and 20) did not suppress the ability to form a DNA-enzyme complex in the presence of MnCl₂. As expected, the IN50–288 protein (lanes 5, 12, and 19) was able to bind to the oligonucleotide substrate probably due to its DNA-binding domain. IN1–55, containing only the N terminus of the protein, displayed a low level of DNA-protein complex of the expected molecular weight (lanes 7, 14, and 21) but also generated a DNA-protein complex having a large molecular weight of unknown identity.

Consistent with earlier results from UV cross-linking (11), an IN50–212 deletion mutant containing only the central catalytic domain was not able to form a Schiff base with the linear duplex oligonucleotide substrates containing an abasic site (Fig. 6B, lanes 4 and 8). This lack of binding, however, was not due to the lack of an appropriately positioned amino group in the central domain because an IN50–212 mutant containing a histidine tag (which contains no lysine residues but allows the deletion mutant to bind to linear DNA) was able to bind to
these same oligonucleotide substrates (Fig. 6B, lanes 3 and 7).

Site-directed mutagenesis and sequence alignment have identified three amino acid residues in the catalytic core, which are conserved among all retroviral integrases (35) and are critical for activity (36). These are Asp-64, Asp-116, and Glu-152. IN116N has previously been shown to bind to the linear substrate and protein concentrations in this experiment were 75 and 80 nM, respectively. DNA interaction. Wild-type integrase was incubated with the viral DNA binding site and residues involved in interactions with both the conserved CA dinucleotide and subterminal sequences.

We determined whether integrase which had formed a Schiff base with the abasic site-containing oligonucleotide substrate was still competent for catalysis, since the chemical cross-linking may be a fairly gentle perturbation of the integrase-DNA interaction. Wild-type integrase was incubated with the oligonucleotide substrate +11 (see Fig. 3A) prior to or concurrent with the addition of borohydride for increasing times. A DNA-enzyme complex was detected using both conditions (data not shown). Furthermore, both 3′-processing and strand transfer products were detected whether borohydride was added after or at the start of incubation (data not shown). No change was observed in the relative extents of glycerolysis, hydrolysis, and circular nucleotide formation (37, 38) when HIV-1 integrase was chemically cross-linked to 3′-end labeled substrates at the start of the reaction versus after the reaction had proceeded (data not shown). We conclude that chemically cross-
linked integrase is competent for catalysis.

Finally, we also tested whether our assay could be used to determine the inhibitory mechanisms of drugs. We preincubated HIV-1 integrase with either of two recently described inhibitors, the guanosine quartet structure formed by the oligonucleotide T30177 (39) or a nonnucleoside inhibitor, the 4-hydroxyxoumarin “butterfly” structure (41), prior to incubation with abasic site-containing oligonucleotide substrates (see Fig. 3A). We found that both inhibitors were able to inhibit the binding of integrase to the DNA substrates (data not shown), consistent with results from UV cross-linking (40, 41).

**DISCUSSION**

The interactions of various proteins with nucleotide or polynucleotide substrates can be characterized by chemically generating an aldehyde which would be able to form an imine upon binding to the enzyme. This strategy was recently exploited using ATP which was first oxidized to the 2',3'-dialdehyde. This modified nucleotide was then incubated with HIV-1 integrase and a Schiff base was detected upon reduction with NaCNBH₃ (21). The nucleotide binding site was further explored using pyridoxal phosphate, an inhibitor of HIV-1 integrase (42), which was also found to bind to integrase via a Schiff base (21). A similar strategy was used in studying the interactions of GTP (which was first oxidized to the 2',3'-dialdehyde) with the α-subunit of the stimulatory G protein rGₛₙ-α (43). Abasic sites have also been created in polynucleotide substrates via chemical reagents and imine formation could then proceed by reaction of an appropriately positioned lysine with the aldehydic abasic site. Such a strategy has been used in defining the interactions between histones and DNA (44).

This is the first report using this technique to probe a substrate DNA-enzyme interaction. This report describes the optimization of conditions and demonstrates the feasibility of this technique to gain further insight into the HIV-1 integrase DNA binding mechanism. Using oligonucleotide substrates of increasing length, we randomly introduced approximately one abasic site per duplex by chemical methods. This random “lesion mutagenesis” allowed us to rapidly screen for the formation of a Schiff base as well as for conditions where substrate length was important. We found that DNA binding through Schiff base formation was higher with manganese than magnesium with a 21-mer duplex substrate. However, this differential level of complex formation was not observed when 35- or 50-mer duplex substrates were used (Fig. 2). These results are consistent with earlier findings describing the effect of substrate length on catalytic activity (24).

The enzymatic introduction of one abasic site per duplex at a known location allowed us to attempt a fine mapping of optimal sites of interaction with the DNA substrate using both wild-type and deletion mutants of HIV-1 integrase and to provide a basis for understanding results from substrate mutagenesis studies (33, 34, 45–47). We found that optimal Schiff base formation occurred with substrates near the conserved CA dinucleotide (Fig. 3), consistent with results obtained from mutagenesis experiments which showed that these two nucleotides and those proximal to them are important for catalysis. These results are also consistent with our data showing that a loop proximal to the processing site diminishes the ability of integrase to bind to the substrate (Fig. 5). An alternative explanation for the wavelike dependence of Schiff base formation on the location of the abasic site could be that one lysine residue is close enough to the processing site such that it acts as the only nucleophile in the Schiff base formation for substrates -1 through -7. However, as the abasic site is moved further away from the processing site (e.g. substrates -5 through -7), this lysine residue becomes too far removed to efficiently form a Schiff base, and a second lysine residue may act as the nucleophile in the Schiff base formation with substrate -11 (and presumably with other substrates in which an abasic site is even further away from the processing site).

The presence of either manganese or magnesium greatly enhanced the level of Schiff base formation, although a low level of an enzyme-DNA complex was generated in the absence of divalent metal ion (Fig. 4), consistent with previous results from UV cross-linking (11). This increased binding in the presence of metal suggests that the metal participates in efficient DNA binding. A novel aspect of the current study is that preformed binary complexes containing integrase and bound divalent metal can be dissociated by the addition of EDTA (Fig. 4, C and D), resulting in a reduction in the level of DNA binding by the enzyme. These data suggest that the metal is loosely bound to the HIV-1 integrase in the absence of DNA, which is consistent with the fact that crystals of ASV integrase required soaking in high concentrations of either MnCl₂ (10 mM) or MgCl₂ (500 mM) in order to obtain clear electron-density maps of crystals of either divalent metal bound to the integrase (27). This is also consistent with the requirement of a high concentration of MnCl₂ (25 mM) for assembly of integrase-donor DNA complexes prior to the strand transfer reaction (30). However, the observation that addition of EDTA after the formation of the enzyme-metal-DNA complex failed to reverse the integrase-DNA complexes (Fig. 4, E and F) suggests the existence of stable ternary integrase-metal-DNA complexes. The existence of such a ternary complex is consistent with structural data from the DNA polymerase model of Beese and Steitz (40).

Site-directed as well as N- and C-terminal deletion mutants were able to form a Schiff base with the oligonucleotides containing an abasic site (Fig. 6), demonstrating that the lysine residue(s) which is involved in imine formation is located in the central catalytic domain. However, the presence of either the N or C terminus was required for efficient DNA binding because the deletion mutant IN50–212 was not able to bind to the linear duplex substrate.

Which lysine residue(s) forms the Schiff base with the abasic site in the DNA? As an initial attempt to address this question we tested a lysine to glutamate mutation at position 136 of HIV-1 integrase. This mutant protein demonstrates catalytic activity approximately equal to that of wild-type HIV-1 integrase in the protein concentration range of 500 nM and only slightly reduced activity at lower concentrations.² This mutant demonstrated significantly reduced although still detectable binding by our assay. At a protein concentration of 80 nM, there was a 4–10-fold reduction (depending on the substrate used) in the level of DNA binding compared to wild-type HIV-1 integrase (Fig. 7). Moreover, the pattern in the level of Schiff base formation as a function of base sequence was altered using this mutant compared to the wild-type. Future mutation studies using this assay are planned to determine which lysine(s) residue comes into contact with the viral DNA substrate and to define the viral DNA binding site(s). In the absence of a crystal structure of integrase bound to its viral DNA substrate, such studies should provide valuable insight into interactions between residues on the integrase and the conserved CA dinucleotide and subterminal sequences.

In summary, we have described a novel and simple assay for monitoring the binding of HIV-1 integrase to its viral DNA

² Mazumder, A., Neamati, N., Ojwang, J. O., Sunder, S., Rando, R. F., and Pommier, Y. (1996) Biochemistry 35, in press.

³ A. Engelman, personal communication.
substrate via the formation of a Schiff base with an enzymatically introduced abasic site. Requirements for Schiff base formation paralleled those for UV cross-linking and catalysis and may provide a basis for understanding substrate mutagenesis and catalysis. We provide experimental evidence for the stability of ternary integrase-metal-DNA complexes but not binary integrase-metal complexes in the presence of EDTA, consistent with a loose binding of metal ion by integrase in the absence of DNA. This novel approach may also provide insights into structural details of integrase interactions with its DNA substrate.

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