Substrate-Length-Dependent Activities of Human Immunodeficiency Virus Type 1 Integrase *in Vitro*: Differential DNA Binding Affinities Associated with Different Lengths of Substrates†

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ABSTRACT: Human immunodeficiency virus type 1 integrase (HIV-IN) is an enzyme essential for the integration of viral DNA into the host chromosome, a process that is an attractive target for drug development. *In vitro* assays have been developed to study both components of the integration process, the 3′-processing and strand transfer reactions. However, major discrepancies between results obtained from *in vivo* and *in vitro* events raise concerns as to the biological relevance of activities observed *in vitro*. These discrepancies include the size of the substrate and the nature of the divalent cation used. In this study, we characterized activities of HIV-IN with oligonucleotide substrates varying in length. Our previous studies indicate that the preferred cation *in vitro* for 3′-processing is altered from Mn^{2+} to Mg^{2+} by increasing the length of the oligonucleotide substrate. This study demonstrates that HIV-IN efficiently catalyzes Mg^{2+}-dependent 3′-processing while repressing the strand transfer reaction. Substrate competition studies indicate that longer substrates preferentially bind to the viral DNA binding site of the integrase, whereas the shorter substrate has much less specificity. In addition, the shorter substrate requires a higher concentration of Mg^{2+}, indicating that there is an alteration in the metal binding affinity associated with the varying substrates. Our results show that substrate-length-dependent differential activities are due to differences in the divalent metal binding and DNA binding affinities associated with the different substrates. These results suggest that the structure of the viral DNA is an important factor in differentiating the donor and target substrates. Characterization of DNA substrates for *in vitro* assays may resolve some of the *in vitro* and *in vivo* discrepancies and provide further understanding of structure/function relationships between IN and DNA.

Retroviral integration is an essential step in the retrovirus life cycle. The advantages of targeting retroviral integration in the search of specific inhibitors are the absence of any known cellular counterparts for HIV-IN,[1] and the availability of *in vitro* assays that facilitate both structural and functional characterizations of the enzyme and the initial screening of specific inhibitors. These *in vitro* assay systems examine 3′ donor processing, strand transfer, and disintegration reactions with short double-stranded oligonucleotides corresponding to either the U5 or U3 ends of HIV-1 DNA and purified IN (Varmus & Brown, 1989; Grandgenett & Mumm, 1990; Katz & Skalka, 1994).

It has been shown previously that nonspecific DNA competes with short oligonucleotide substrates for binding to HIV-IN in the presence of Mn^{2+} (van Gent et al., 1991) and that this nonspecific DNA is also cleaved at a low efficiency by the enzyme. In addition, it has been reported that plasmid DNA is nicked preferentially in the presence of Mn^{2+} rather than Mg^{2+} (Sherman & Fyfe, 1990). As demonstrated in the accompanying paper (Lee et al., 1995), our results show that HIV-IN exhibits several differences in activity depending on the length of the substrates and the cation utilized: (1) In general, Mn^{2+}-dependent 3′-processing activity is associated with shorter oligonucleotides. However, the Mg^{2+}-dependent activity is increased with an increase in the length of the substrates. (2) In the presence of Mn^{2+}, integrase is able to use the same short substrate as *both donor and target* in the strand transfer reaction. This reaction is significantly reduced in the presence of Mg^{2+}. (3) Disintegration reactions are primarily observed in the presence of Mn^{2+} and significantly diminished with Mg^{2+}. Interestingly, it has been previously reported that mutant integrase (IN 50-212), which does not catalyze donor processing or strand transfer, mediates the disintegration reaction in the presence of Mn^{2+} (Engelman & Craigie, 1992; Vincent et al., 1993; Engelman et al., 1993). (4) DNA substrates which contain additional single-strand target sequences at the 3′ end of the processing strand improve the efficiency of the cleavage reaction. These results suggest that Mn^{2+} is associated with less specific reactions, while Mg^{2+} represses these activities.

We propose that structural elements associated with longer viral DNA substrates are factors in determining the specificity of DNA and metal binding and that divalent cations, DNA-induced subunit interactions, and conformational changes associated with protein/DNA interaction have additional roles in the overall integration process. We postulate that (1) the sequence within the U5 LTR confers structural elements determining the specificity of DNA binding, (2) the affinity

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; IN, integration protein (integrase); PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair; ATP, adenosine 5′-triphosphate.

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Chart 1

Substrate 1:	5'-CCC**GTGTGGAAAATCTCTAGCAGT**
(U5 24/24)	3'-GGG**CACACCTTTTAGAGATCGTCA**
Substrate 2:	5'-**TTTAGTCAGTGTGGAAAATCTCTAGCAGT**
(U5 29/29)	3'-**AAATCAGTCACACCTTTTAGAGATCGTCA**
Substrate 3:	5'-**GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT**
(U5 35/35)	3'-**CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA**
Substrate 4:	5'-CCC**GTGTGGAAAATCTCTAGCA**
(U5 22/24)	3'-GGG**CACACCTTTTAGAGATCGTCA**
Substrate 5:	5'-GCAGGGCGAAAGCGACCGCGCC
(22/22)	3'-CGTCCCGCTTTCGCTGGCGCGG

of Mg^{2+} binding is defined by a sequence-dependent protein/DNA complex, and (3) HIV-IN binds both sequence-specific viral DNA (for 3′-processing) and nonspecific DNA (for the strand transfer reaction).

In this study, we characterize both the 3′-processing and strand transfer activities of HIV-IN with substrates varying in length (24-, 29-, and 35-bp DNA) to test these hypotheses. We demonstrate that the 35-bp DNA substrate preferentially binds to the specific viral DNA binding site as compared to the 24-bp DNA substrate and that the efficiency of the Mg^{2+}-dependent 3′-processing activity increases as the substrate length increases. This may be an important observation in resolving *in vivo* and *in vitro* discrepancies and in understanding the structure/function relationships of HIV-IN and DNA.

MATERIALS AND METHODS

Protein Purification. Plasmid pINSD containing the gene encoding HIV-1 IN was obtained from Dr. R. Craigie and overexpressed in *Escherichia coli* (BL21-DE3). The wild-type integrase was purified according to the procedure previously described (Sherman & Fyfe, 1990).

Oligonucleotide DNA Substrates. Oligonucleotides containing the terminal sequence of HIV-1 DNA were purchased from Integrated DNA Technologies, Inc. The sequences of the oligonucleotides are shown in Chart 1. Bold letters indicate sequences of the U5 end of the HIV-1 DNA. U5 24/24, 29/29, and 35/35 represent the blunt, unprocessed end of the U5 *att* site of HIV-1. U5 22/24 represents the preprocessed end of the U5 *att* site. Substrate 5 is a nonspecific DNA. Oligonucleotides were purified by electrophoresis through 20% polyacrylamide (29:1 acrylamide: bisacrylamide) denaturing gels (7 M urea). The oligonucleotide samples were electroeluted from the sliced gels using the S&S Elutrap electroseparation system from Schleicher & Schuell and ethanol-precipitated.

Assays for IN Activities. One microgram of the appropriate oligonucleotide was ^{32}P-labeled at the 5′ termini with T4 polynucleotide kinase (New England Biolabs) and 25 μCi of adenosine [γ-^{32}P]-5′-triphosphate (3000 Ci/mmol, ICN). The labeled oligonucleotide was separated from the unincorporated [γ-^{32}P]ATP using a Sephadex G-25 Quick Spin column (Boehringer Mannheim). The labeled oligonucleotide was then annealed with a 3-fold molar excess of unlabeled complementary strand in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 M NaCl. All the reaction mixtures for the IN protein-mediated cleavage reactions contained 25 mM HEPES, pH 7.5, 2.5 mM DTT, 50 mM NaCl, 5% glycerol (v/v), 0.05% NP-40, 0.5 mM CHAPS, 7.5 mM Mg^{2+} or 7.5 mM Mn^{2+}, ^{32}P-labeled substrates (typically 0.5 pmol), and 5 pmol of HIV-1 IN in a total volume of 20 μL. The reactions were initiated by the addition of IN and incubated for 60 min at 37 °C. The reactions were stopped by the addition of an equal volume of stop solution (95% formamide, 30 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) and the reaction samples were boiled for 5 min. A 10-μL of aliquot of each reaction mixture was electrophoresed on a 7 M urea denaturing 15% or 20% polyacrylamide sequencing gel and the reaction products were analyzed by autoradiography. Quantitation of 3′-processing and strand transfer reaction products was performed with a Pdi densitometer Model DNA 35 using a Kodak photographic step tablet for the calibration curve.

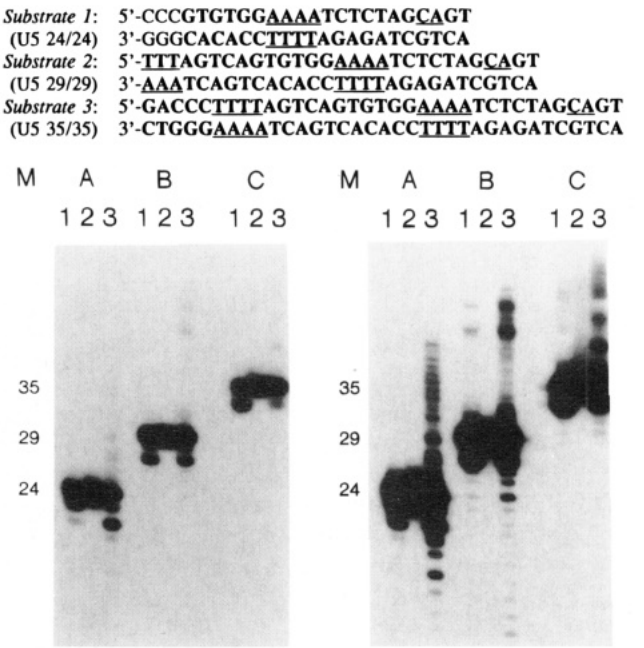

FIGURE 1: Substrate-length dependence on the activities of HIV-IN. The left panel shows the 3′-processing reaction performed with three radiolabeled U5 LTR oligonucleotide substrates varying in length and wild-type HIV-1 integrase. (A), (B), and (C) are the 24-, 29-, and 35-bp substrates (*substrates 1, 2, and 3*), respectively. In A−C, lanes 1 and 3 represent reactions containing the respective substrates and wild-type integrase with 7.5 mM $MgCl_2$ and 7.5 mM $MnCl_2$, respectively. Lane 2 is the control lane which does not contain HIV-IN. The right panel is an overexposure of the same gel to show better the strand transfer reactions. Unless otherwise noted, each reaction was performed as previously described in Materials and Methods.

RESULTS

Effects of the DNA Substrate Length on 3′-Processing and Strand Transfer Reactions of HIV-IN. To determine whether the sequence within the U5 LTR of HIV-1 DNA affects the activities of HIV-IN, three DNA substrates of varying lengths were prepared and activities with Mg^{2+} or Mn^{2+} were characterized. The sequences of these substrates (24, 29, and 35 bp) correspond to the U5 end of HIV-1 DNA. The 3′-processing and strand transfer activities of HIV-IN with these substrates in the presence of Mg^{2+} or Mn^{2+} as the divalent cation are shown (Figure 1). The cleavage reactions of the 24-bp substrate 1 showed a cleavage efficiency of 26% with Mn^{2+} as compared to 3.8% cleavage with Mg^{2+} (Figure 1, left panel, lanes A). Although in this figure the Mg^{2+} had a low cleavage efficiency, we have observed that the Mg^{2+}-dependent 3′-processing activity of this substrate can vary in cleavage efficiency from 10% to 20% depending on the reaction conditions used. However, under identical reaction conditions Mn^{2+} promotes higher amounts of

cleavage products than reactions with Mg^{2+}. This preferential activation by Mn^{2+} has been previously observed (Sherman & Fyfe, 1990; Craigie et al., 1990). The efficiencies of cleavage of the 29-bp substrate 2 were 25% with Mn^{2+} and 29% with Mg^{2+} (Figure 1, lanes B). These results indicate that the efficiency of Mn^{2+}-dependent activity remains the same while the Mg^{2+}-dependent activity is substantially increased as the length of the substrate is increased from 24 to 29 bp. When the 35-bp substrate 3 was examined, the extents of the cleavage were 13% with Mn^{2+} and 38% with Mg^{2+} (Figure 1, lanes C), indicating that as the substrate is further increased in length there is an alteration in the preferred cation from Mn^{2+} to Mg^{2+}. This differential activity is consistent with the endonucleolytic activity demonstrated in the accompanying paper (Lee et al., 1995).

These substrates also exhibited additional differences in terms of their strand transfer reactions. As the substrate was increased in length, the efficiency of the strand transfer reaction was slightly enhanced with Mg^{2+}. This trend could be attributed to the inefficiency of the respective 3′-processing reactions. However, although the 29- and 35-bp substrates showed more efficient cleavage with Mg^{2+} versus Mn^{2+}, the efficiency of strand transfer was considerably lower in the Mg^{2+} reactions than the Mn^{2+} reactions (Figure 1, right panel). Therefore, differences in strand transfer can be attributed to differences in the substrate and not to the efficiency of the 3′-processing reaction. Furthermore, the patterns of strand transfer products were also different with respect to substrates. If the Mn^{2+} reactions are compared, the 24-bp substrate 1 exhibited random sites of integration whereas integration with the two longer substrates were more selective. These results indicate that HIV-IN displays a differential activity associated with substrate length.

The significance of these results is that the differential activity of HIV-IN accompanies an apparent alteration in the preferred cation depending on the substrate length and the 3′-processing activity in Mg^{2+} is enhanced as the length of the viral donor substrates increases. This may explain why Mg^{2+}-dependent 3′-processing activity has not been previously observed (Sherman & Fyfe, 1990; Craigie et al., 1990). Moreover, consistent with previously reported results (Drelich et al., 1992), the Mg^{2+}-dependent reactions display inefficient strand transfer activity, indicating that Mg^{2+} does not promote the strand transfer reactions into viral LTR sequences. Inefficient strand transfer using the preprocessed substrates with Mg^{2+} showed similar results (Figure 7). This implies that the 3′-processing and strand transfer reactions are not directly linked. Overall, these results lead us to conclude that Mn^{2+} is not the preferentially required cofactor for 3′-processing and that the sequences within the longer LTR substrates may play an important role in discriminating the affinity for the two different divalent cations.

Metal Concentration Dependency on the 3′-Processing Activity of HIV-IN. To assess the affinities of the two divalent cations, the 3′-processing activity of HIV-IN was characterized as a function of metal cation concentration. The results shown in Figure 2 (top) represent the autoradiographic data obtained from the 29-bp DNA substrate and the bottom panel represents the quantitated data obtained by scanning the autoradiograph. Although both divalent cations display 30% cleavage at 7.5 mM, 1 mM Mn^{2+} showed 29% cleavage of the substrate while Mg^{2+} showed 14% cleavage

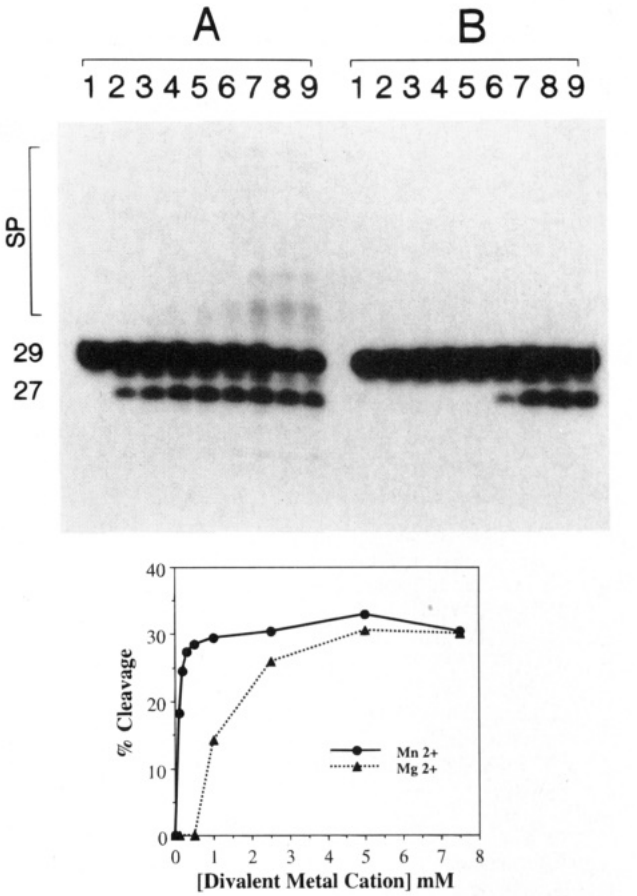

FIGURE 2: Metal concentration dependency on 3′-processing of HIV-IN. The 3′-processing reactions were carried out in the presence of wild-type HIV-IN, the 29-bp U5 LTR oligonucleotide substrate (substrate 2, 5′-TTTAGTCAGTGTGGAAAATCTCTAG-CAGT annealed to 3′-AAATCAGTCACACCTTTTAGAGATCGT-CA), and varying concentrations of $MnCl_2$ or $MgCl_2$. (Top) 3′-Processing reactions separated by a 20% denaturing polyacrylamide gel and analyzed by autoradiography. (A) Reactions performed in the presence of $MnCl_2$. (B) Reactions performed in the presence of $MgCl_2$. The concentrations of divalent metal cations used in each series of reactions were 0, 0.1, 0.2, 0.3, 0.5, 1.0, 2.5, 5.0, and 7.5 mM (lanes 1−9, respectively). (Bottom) Quantification of the autoradiograph with a Pdi densitometer Model DNA 35. (●) With Mg^{2+}, (▲) with Mn^{2+}.

(Figure 2, bottom). These results indicate that the apparent binding affinities for the divalent cations are not the same. Although it is not possible to determine precise dissociation constants for Mn^{2+} and Mg^{2+} from these experiments, it is apparent that the binding affinity is substantially higher for Mn^{2+} than for Mg^{2+}. These results may further explain why previous studies found 3′-processing activity at 1 mM Mn^{2+} but not at 1 mM Mg^{2+} (Sherman & Fyfe, 1990).

pH Dependency on the 3′-Processing Activity of HIV-IN. To determine the effects of pH, the 3′-processing activity of the three substrates were characterized as a function of pH. When the pH of the reactions was titered from 6.3 to 8.3 utilizing HEPES buffer, the Mg^{2+}-activated reactions were found to be optimal at pH 7.5 for both the 29-bp (36% cleavage) and 35-bp (44% cleavage) DNA substrates (Figure 3). Both substrates displayed little activity with Mg^{2+} at pH 8.0. In contrast, the Mn^{2+}-activated reactions displayed optimal activity at pH 8.0 for both the 24-bp (34% cleavage) and 29-bp (34% cleavage) DNA substrates (Figure 3). The efficiency of the 3′-processing reaction decreased progressively at a lower pH which was expected since metal

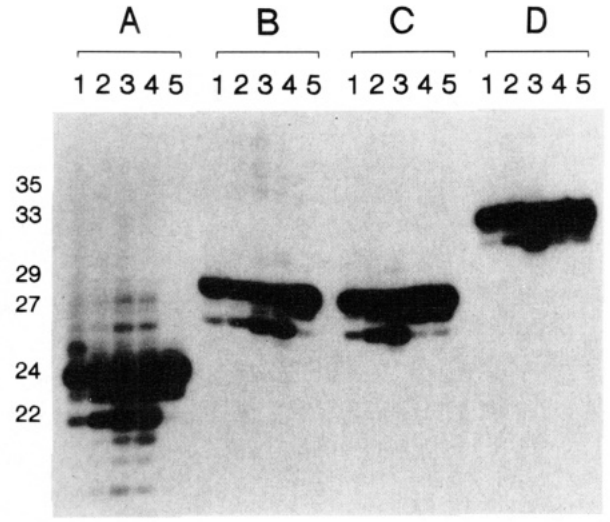

FIGURE 3: pH-dependent 3′ donor processing activity of HIV-IN. The 3′-processing reactions were carried out with various substrates and their preferred divalent cations at varying pH with wild-type HIV-IN. (A) U5 LTR substrate (24 bp) (*substrate 1*, 5′-CCCGTGTGGAAAATCTCTAGCAGT annealed to 3′-GGGCACACCTTTTAGAGATCGTCA) in 7.5 mM $MnCl_2$. (B) U5 LTR substrate (29 bp) (*substrate 2*, 5′-TTTAGTCAGTGTGGAAAATCTCTAGCAGT annealed to 3′-AAATCAGTCACACCTTTTAGAGATCGTCA) in 7.5 mM $MnCl_2$. (C) U5 LTR substrate (29 bp) (*substrate 2*) in 7.5 mM $MgCl_2$. (D) U5 LTR substrate (35 bp) (*substrate 3*, 5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT annealed to 3′-CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA) in 7.5 mM $MgCl_2$. In each series of reactions, lanes 1−5 represent reactions performed with 25 mM HEPES buffer at pHs of 6.3, 7.0, 7.5, 8.0, and 8.3, respectively. All reactions were performed as previously described in Materials and Methods unless otherwise noted.

cofactor(s) bind to the carboxylic groups of acidic amino acids (Winkler et al., 1993). A significant difference was noticed when the Mn^{2+}-dependent activity of the 24-bp substrate was compared to the Mg^{2+}-dependent activity of the 35-bp DNA substrate. The 35-bp substrate displays a very specific pH requirement for optimal reaction conditions, while the 24-bp DNA substrate tolerates wider pH ranges, indicating that specific 3′-processing by HIV-IN requires Mg^{2+}. Thus, the pH of the reaction conditions further differentiates the discrete differences between the reactions activated by the two divalent cations.

Preferentially Cleaved Substrate in the Presence of Competing Substrate. The differential cleavage activities displayed by these substrates make it possible to determine the functional roles of the two divalent cations and the effects of the length of the DNA substrates. To determine whether the substrate-length-dependent differential activities are due to differences in the metal or DNA binding affinities of the different length of substrates, the 3′-processing reactions were characterized as a function of the divalent cations and the competing substrate concentrations.

To determine differences in Mg^{2+} binding affinities associated with the different substrates, the 3′-processing activities of the 24- and 35-bp DNA substrates were assessed as a function of Mg^{2+} concentration. An autoradiograph of the cleavage reactions of each of the substrates is shown in Figure 4 (top). The cleavage products were quantitated using a densitometer and the results were plotted (Figure 4, bottom). The 35-bp substrate shows virtually the same cleavage products at 2.5 and 7.5 mM Mg^{2+}. However, the extent of cleavage for the 24-bp substrate was less at 2.5

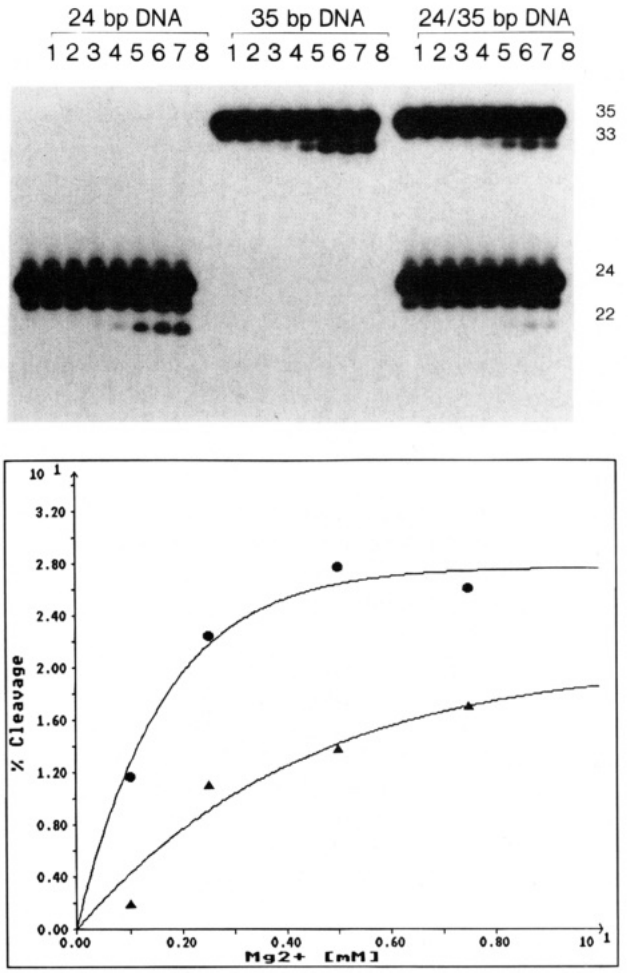

FIGURE 4: Preferentially cleaved substrate in the presence of Mg^{2+}. (Top) Reactions were performed with wild-type HIV-1 IN and each substrate as indicated: 0.5 pmol of 24-bp U5 LTR (*substrate 1*, 5′-CCCGTGTGGAAAATCTCTAGCAGT annealed to 3′-GGGCACACCTTTTAGAGATCGTCA), 0.5 pmol of 35-bp U5 LTR (*substrate 3*, 5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT annealed to 3′-CTGGGAAAATCA GTCACACCTTTTAGAGATCGTCA), and a mixture of 0.5 pmol of 24-bp U5 LTR (*substrate 1*) with 0.5 pmol of 35-bp U5 LTR (*substrate 3*). The $MgCl_2$ concentrations used were 0.1, 0.2, 0.3, 0.5, 1.0, 2.5, 5.0, and 7.5 mM (lanes 1−8, respectively). (Bottom) Quantification of the cleavage reactions with the individual substrates. (●) U5 LTR (35 bp) (*substrate 3*); (▲) U5 LTR (24 bp) (*substrate 1*).

than at 7.5 mM Mg^{2+}. It appears that the 24-bp DNA requires more than 7.5 mM Mg^{2+} for optimal cleavage. The results show that the affinity of Mg^{2+} is higher with the 35-bp DNA substrate than with the 24-bp DNA, indicating that the reactivity of specific metals is dependent on the length of the substrates. This result suggests that a length-dependent DNA/enzyme complex may influence the affinity of Mg^{2+} binding.

To determine whether one substrate can be preferentially cleaved in the presence of the other substrate, the Mg^{2+}-dependent 3′-processing activity of radiolabeled substrates was characterized at pH 7.5. A mixture with equal concentrations of both the 24-bp substrate (0.5 pmol) and the 35-bp substrate (0.5 pmol) was incubated with 5 pmol of HIV-IN as a function of Mg^{2+} concentration and the efficiencies of the 3′-donor processing activity were assessed. The results indicate that the 35-bp substrate was preferentially cleaved, although the cleavage efficiencies of both substrates were reduced due to the increase in the overall substrate concen-

tration. A comparison of these results to those obtained from the 24- and 35-bp DNA substrates indicate that the maximal cleavage of the 35-bp substrate was reduced from 27% to 17% while that of the 24-bp substrate was reduced from 15% to 2% (Figure 4, top). These results suggest that the two substrates have different affinities for the divalent cations and/or different DNA binding affinities, although the results do not discriminate between these differences. To further distinguish the two possibilities, the dissociation constants of each divalent metal cation and DNA binding affinity must be precisely and separately determined. In this current study, we focused on characterizing how DNA binding affinities were affected by the length of the substrates. Further characterization of the differences in the metal binding affinities will be the focus of future studies.

Characterization of Length-Dependent DNA Binding Affinities by Substrate Competition. The results of preferential cleavage (Figure 4) make it possible to perform substrate competition experiments to determine if the differential cleavage patterns are due to different DNA binding affinities for the different lengths of the substrates. The substrates utilized in this study are "partial" competitive inhibitors to each other, because they can bind to the enzyme and they allow for the formation of product at either a reduced or accelerated rate. Competition between substrates for the same DNA binding site is assessed by determining the efficiency of cleavage at a fixed level of radiolabeled substrate with varying concentrations of competing unlabeled substrate. Increasing concentrations of either unlabeled nonspecific 22-bp or specific 24- or 35-bp DNA substrates were added to a fixed concentration of the radiolabeled 24- or 35-bp substrates in the presence of either Mn^{2+} or Mg^{2+}. Competition between the same substrates serves as the control (i.e., radiolabeled 24-bp DNA substrate competes with unlabeled 24-bp DNA).

Previous studies have shown that nonspecific DNA can compete for the shorter DNA substrate (van Gent et al., 1991). To determine whether the nonspecific DNA competes with the 24 bp substrate in the same manner as the 35 bp DNA substrate, both radiolabeled 24- and 35-bp substrates were competed with increasing concentrations of unlabeled nonspecific 22-bp substrate (substrate 5) in the presence of 7.5 mM Mg^{2+}. The results of these experiments are shown in Figure 5. The radiolabeled 24- and 35-bp substrates (0.5 pmol) were reacted with 5 pmol of HIV-IN in a total reaction volume of 20 μL. The concentrations of the competing substrate 5 were 0.5, 1, 2, 4, 8, and 16 pmol. As shown in Figure 5A, increasing concentrations of the competing nonspecific substrate progressively reduced the cleavage reactions of the 24-bp substrates, which is consistent with previous data (van Gent et al., 1991). In contrast, the same concentrations of the competing substrate did not alter the efficiency of the cleavage reactions of the 35-bp DNA substrate. Similar results were obtained from reactions performed in the presence of Mn^{2+} (data not shown). These results suggest that the longer substrate (35-bp DNA) has a higher specificity for HIV-IN, while the shorter substrate (24-bp DNA) has less specificity.

To further confirm these results, each of the radiolabeled 24- and 35-bp substrates was competed with the unlabeled 24- and 35-bp substrates with either Mg^{2+} or Mn^{2+}. The products of the cleavage reactions with the two different divalent cations were separated by gel electrophoresis and

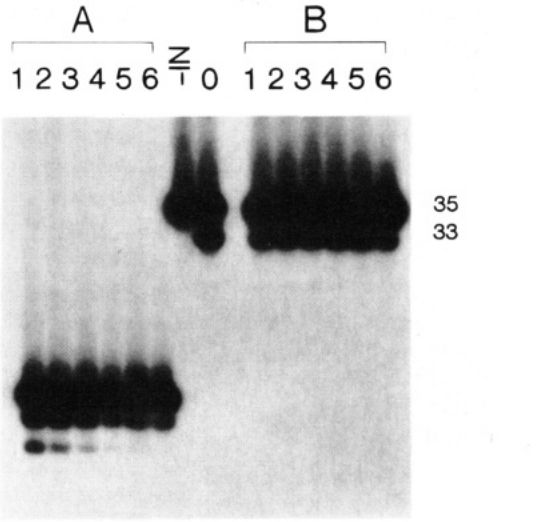

FIGURE 5: Substrate competition between a U5 LTR substrate and nonspecific substrate assessed by the 3′-processing activity of HIV-IN. (A) (0.5 pmol, 24 bp) U5 LTR (*substrate 1*, 5′-CCCGTGTGGAAAATCTCTAGCAGT annealed to 3′-GGGCACACCTTTTAGAGATCGTCA), and (B) U5 LTR (0.5 pmol, 35 bp) (*substrate 3*, =5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT annealed to 3′-CTGGGAAAATCA GTCACACCTTTTAGAGATCGTCA) were individually competed with increasing concentrations of nonspecific DNA (*substrate 5*, 5′-GCAGGGCGAAAGCGACCGCGCC annealed to 3′-CGTCCCGCTTTCGCTGGCGCGG) in the presence of 7.5 mM $MgCl_2$. Lanes 1 and 2 represent the control experiments in the absence and presence of 5 pmol of HIV-IN, respectively. Lanes 3−8 represents reactions with 0.5, 1, 2, 4, 8, and 16 pmol of *substrate 5*, respectively, in a total volume of 20 μL.

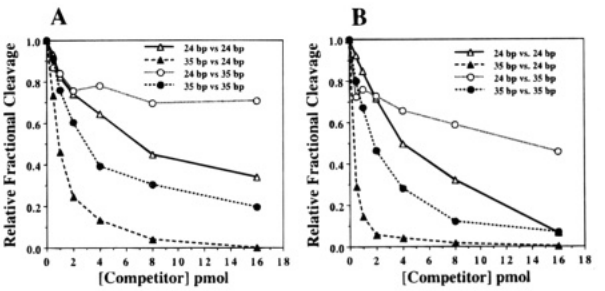

FIGURE 6: Substrate competition between two U5 LTR substrates assessed by 3′-processing of HIV-IN. Substrate competition experiments were performed with 7.5 mM $MnCl_2$ (A) and 7.5 mM $MgCl_2$ (B). The ^{32}P-labeled substrates were fixed at 0.5 pmol and the concentrations of the unlabeled competitors were 0, 0.5, 1, 2, 4, 8, and 16 pmol in 20-μL reaction volumes. (△) Unlabeled 24-bp U5 LTR (*substrate 1*, 5′-CCCGTGTGGAAAATCTCTAGCAGT annealed to 3′-GGGCACACCTTTTAGAGATCGTCA) with radiolabeled *substrate 1*; (▲) unlabeled 35-bp U5 LTR (*substrate 3*, 5′-GACCCTTTTAGTCAGTGTGGAAAATCTC TAGCAGT annealed to 3′-CTGGGAAAATCA GTCACACCTTTTAGAGATCGTCA) with radiolabeled *substrate 1*; (○) unlabeled 24-bp U5 LTR (*substrate 1*) with radiolabeled *substrate 3*; (●) unlabeled 35-bp U5 LTR (*substrate 3*) with radiolabeled *substrate 3*.

the data obtained from scanning the autoradiograph are plotted in Figure 6. "Relative fractional cleavage" represents the ratio of the cleavage products with and without the presence of competing substrates. When a fixed concentration of the radiolabeled 24-bp substrate was competed with the unlabeled 24-bp substrate with 7.5 mM Mn^{2+}, the efficiency of the 3′-processing activity was progressively reduced due to the increasing concentrations of unlabeled substrate. However, the cleavage reactions of the 24-bp substrate showed stronger inhibition when identical concen-

trations of the 35-bp DNA were used as competing substrates (Figure 6A). The extents of cleavage for the control experiments in the absence of competing substrates were 26% with the 24-bp substrate and 19.5% with the 35-bp substrate. As compared to the control, 4 pmol of 35-bp unlabeled substrate reduced 87% of the cleavage reaction of the radiolabeled 24-bp substrate (0.5 pmol), whereas a reduction of 46% was observed with 4 pmol of the 24-bp unlabeled substrate. The stronger inhibition observed by the competing 35-bp substrate indicates that the 35-bp substrate has a higher DNA binding affinity for the enzyme than the 24-bp substrate. Consistent with these results, significant inhibition of the cleavage reactions was not detected when the radiolabeled 35-bp substrate was competed with the unlabeled 24-bp DNA. Four picomoles of the unlabeled 24-bp substrate resulted in a 22% reduction in the cleavage of the 35-bp radiolabeled substrate (0.5 pmol), whereas the same 4 pmol of the unlabeled 35-bp substrate reduced cleavage by 61% (Figure 6A). These results suggest that the 35-bp DNA substrate has a higher DNA binding affinity than the 24-bp DNA substrate, which is consistent with the results shown in Figure 5.

Similar cleavage patterns were observed when the same experiments were repeated in the presence of Mg^{2+} (Figure 6B), confirming the previous data observed with Mn^{2+}. One significant difference between the results obtained with Mn^{2+} versus Mg^{2+} is that the overall inhibition of the cleavage reactions by the presence of competing substrates was slightly higher with Mg^{2+} than with Mn^{2+}. In the absence of competing substrates, the control experiments displayed an extent of cleavage of 20% for the 24-bp substrate and 33% for the 35-bp substrate. As compared to the control, 4 pmol of the 35-bp unlabeled substrate reduced 96% of the cleavage reaction of the radiolabeled 24-bp substrate (0.5 pmol), whereas only a 50% reduction in cleavage was observed with 4 pmol of the 24-bp unlabeled substrate (Figure 6B). Four picomoles of the unlabeled 24-bp substrate resulted in a 25% reduction in the cleavage of the 35-bp radiolabeled substrate (0.5 pmol), whereas 4 pmol of the unlabeled 35-bp substrate reduced 72% of the cleavage (Figure 6B). These results further imply that the DNA binding affinity to the viral donor DNA binding site is enhanced by the presence of Mg^{2+}.

To further examine whether the differences in DNA binding affinity associated with different lengths of substrate have an effect on the strand transfer activity of HIV-IN, the preprocessed (CA-3′) radiolabeled substrate 4 was competed with unlabeled 24- or 35-bp substrates. Since the strand transfer activity of HIV-IN also depends on the efficiency of 3′-processing activity, the preprocessed substrate is used to separately assess the effects of different substrates on the strand transfer reaction. The control experiments were performed with the preprocessed DNA substrate in the presence of Mg^{2+} or Mn^{2+} and without competing substrate. The results of these experiments are shown in Figure 7. As previously observed (Figure 1), the strand transfer products were significantly reduced in the presence of Mg^{2+} as compared to Mn^{2+} (Figure 7C). When the 24-bp unlabeled substrate was used as the competing substrate, the strand transfer activities of the radiolabeled substrate 4 were progressively reduced as observed in the 3′-processing activities with Mn^{2+} (Figure 7B), suggesting that the unlabeled substrate competes for the donor binding site. However, a significantly low efficiency of strand transfer

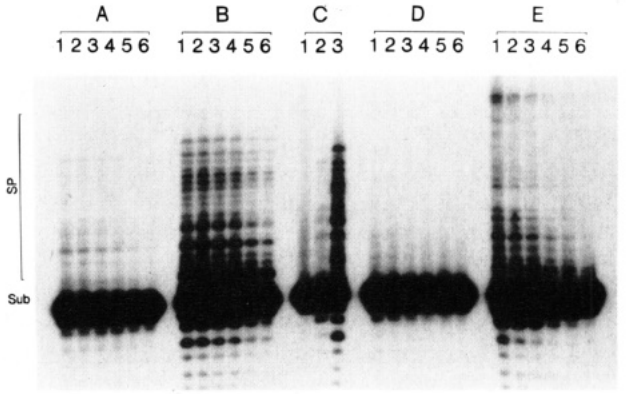

FIGURE 7: Substrate competition between the U5 LTR substrates assessed by the strand transfer reaction of HIV-IN. The ^{32}P-labeled preprocessed U5 LTR (*substrate 4*, 5′-CCCGTGTGGAAAATCTCTAGCA annealed to 3′-GGGCACACCTTTTAGAGATCGTCA) was fixed at 0.5 pmol and the concentrations of the unlabeled competitors were 0, 0.5, 1, 2, 4, 8, and 16 pmol (lanes 1−6, respectively) in 20-μL reaction volumes. Panels A and B are reactions with unlabeled *substrate 1* (5′-CCCGTGTGGAAAATCTCTAGCAGT annealed to 3′-GGGCACACCTTTTAGAGATCGTCA) in 7.5 mM $MgCl_2$ and 7.5 mM $MnCl_2$, respectively. Panels D and E are reactions with unlabeled *substrate 3* (5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGT annealed to 3′-CTGGGAAAATCA GTCACACCTTTTAGAGATCGTCA) in 7.5 mM $MgCl_2$ and 7.5 mM $mNCl_2$, respectively. Panel C represents the control experiments in the absence of competing substrates: lane 1, no HIV-IN; lane 2, with HIV-IN in 7.5 mM $MgCl_2$; lane 3, with HIV-IN in 7.5 mM $MnCl_2$.

activities was observed with Mg^{2+} (Figure 7A). Likewise, when the 35-bp substrate was competed, stronger inhibition of the strand transfer activities was observed in the presence of Mg^{2+} as compared to those obtained with the 24-bp competing DNA. An additional difference is that the strand transfer products were longer with the 35-bp competing DNA than with the 24-bp substrate, indicating that the 35-bp substrate could be used as a target with Mn^{2+} (Figure 7E). In the presence of Mg^{2+}, the 35-bp competing substrate produced very small amounts of strand transfer products that were different than those obtained with the 24-bp competing substrate. Overall, the efficiency of the strand transfer was less with the 35-bp DNA as the competing substrate than with the 24-bp substrate (Figure 7D,E). This suggests that the 35-bp competing substrate has a higher DNA binding affinity to the viral DNA binding site than the preprocessed radiolabeled DNA substrate 4, thereby inhibiting the strand transfer activity.

Association of different lengths of substrate with different DNA binding affinities does not entirely explain the low efficiency of strand transfer with Mg^{2+}. Therefore, divalent cations must have another role. It is possible that Mg^{2+} may play an important role in DNA binding to both the donor and target DNA binding sites: Mg^{2+} may increase the binding affinity of the longer substrate to the viral donor DNA, thereby promoting the 3′-processing activity and suppressing the binding of the viral donor substrates to the sequence-nonspecific target binding site on the enzyme, thus inhibiting the strand transfer activities. In contrast, Mn^{2+} does not appear to discriminate by substrate specificity. Therefore, Mg^{2+} may further strengthen the specificity of reactions mediated by HIV-IN, while Mn^{2+} may allow integrase to tolerate substrate specificity for the integration reaction. The ability of Mn^{2+} to lower the stringency of enzyme-catalyzed reactions has been reported for other

enzymes: DNA polymerase (Loeb & Kunkel, 1982), restriction enzyme *Eco*RV (Vermote & Halford, 1992), and *E. coli* Holliday junction resolvase RuvC (Shah et al., 1994).

DISCUSSION

Subunit interactions, structural elements within the viral DNA, and divalent cations are important in determining sequence-specific vs non-specific DNA interactions and the catalytic activity of HIV-IN. The main objective of this study is to address the following questions: (1) How does HIV-IN specifically recognize both target and viral donor substrates? (2) What is the differential role of the divalent metal cofactor(s)? We characterized the activities of HIV-IN utilizing various oligonucleotide substrates with different lengths in the presence of either Mg^{2+} or Mn^{2+} and found that increasing the length of the substrate resulted in differential effects on 3′-processing activity of HIV-IN *in vitro*. More importantly, the Mg^{2+}-dependent activity of HIV-IN does not efficiently promote the strand transfer reaction, consistent with the *in vitro* results obtained from endogenous viral DNA. It has been previously reported that integrase catalyzes the integration of endogenous viral DNA as part of a nucleoprotein complex but not naked DNA in the presence of Mg^{2+} (Brown et al., 1987; Bowerman et al., 1989).

3′-Processing Activity of HIV-IN Displays Substrate Length Dependency. Amino acid sequence comparisons of retroviral integrase proteins and mutational analyses of integrases have identified the central core region conserved in the transposases of certain bacterial transposons (Fayet et al., 1990; Kulkosky et al., 1992; Rowland & Dyke, 1990). This conserved region is located approximately between amino acids 60 and 160 in the protein and consists of three acidic amino acids in the arrangement D-D-35XX-E (Fayet et al., 1990; Kahn et al., 1991). The function of these acidic amino acids is to bind two divalent cations. Mutations of any of these residues lead to the inactivation of IN, suggesting that divalent cations are catalytically important for the integrase protein and that the central core is critical for catalysis of polynucleotidyl transfer (Engelman & Craigie, 1992; Drelich et al., 1992; Kulkosky et al., 1992; Vincent et al., 1993; Vink et al., 1993).

All bacterial transposons contain these three acidic amino acids and require Mg^{2+} as their metal cofactor. In contrast, the donor processing reaction for HIV-1 IN characterized with a short oligonucleotide substrate is catalyzed with a preference for Mn^{2+} over Mg^{2+} (Sherman & Fyfe, 1990; Vincent et al., 1993). This characteristic has been observed with other retroviral IN proteins. MoMLV and avian IN proteins are much more efficient in cleaving the viral DNA with Mn^{2+}, but the avian IN protein maintains a greater selectivity in the presence of Mg^{2+}. This implies that Mg^{2+} is required for optimal specificity even though the activity is reduced (Katzman et al., 1989). Our results demonstrate that HIV-IN can efficiently catalyze the Mg^{2+}-dependent sequence-specific cleavage of DNA substrates, similar to those previously reported (Drelich et al., 1992). Furthermore, the efficiency of the Mg^{2+}-dependent 3′-processing activity of HIV-IN was enhanced as the length of the substrate was increased. These differential activities are apparently due in part to the different DNA binding and metal binding affinities associated with the length of DNA substrates. How can the effects of substrate-length-dependent differential activities of HIV-IN be explained?

Both the length and local conformation of a DNA molecule can have a profound influence on protein−DNA interactions because the structure of the DNA double helix is not monotonous and instead exhibits marked sequence-dependent variation. For example, if a protein recognizes a stable structure as a bend, then specificity would be enhanced by the presence of the specific sequences allowing the DNA bending. DNA bending is in fact a frequent phenomenon which contributes to specificity in protein−DNA interactions and DNA-induced protein−protein interactions (Schultz et al., 1991). We hypothesize that the observed differential activities of HIV-IN may be attributed to the length-dependent structure of the DNA substrate itself. The structural elements within the DNA substrate determine specificity of DNA binding to the enzyme and induce the correct DNA/protein complex. The shorter oligonucleotide substrates, being unable to fill the DNA binding site in the protein, may not induce conformational changes in protein and/or DNA following their interaction.

It has previously been reported that, in the recombination of bacteriophage λ, AAAA tracts can be inserted into the substrate, allowing the DNA to bend and thereby providing the proper conformation for DNA binding and functionally replacing the need for integration host factor (Goodman et al., 1992). Interestingly, the U5 LTR sequence of the endogenous viral DNA contains a second AAAA tract when the conventioanl 21-bp substrate is extended to 35-bp. The longer substrate (35 bp) has a much higher DNA binding affinity to HIV-IN compared to the shorter substrate (24 bp) (Figure 6). Nonspecific DNA was not able to compete with the longer substrate (Figure 5). Therefore, the Mg^{2+}-dependent reaction observed with longer substrates may be explained by the additional AAAA tracts altering the DNA binding affinity of the enzyme. Furthermore, the presence of DNA bending may influence the specificity of DNA/protein interaction. Further characterization of the effects on the activities of HIV-IN of these AAAA tracts located within the U5 viral LTR will provide a better understanding of substrate sequence requirements for *in vitro* assay systems.

Specific vs Nonspecific DNA Binding. Viral integration requires two separate steps: the specific 3′ donor processing, which occurs in the cytoplasm, and the nonspecific strand transfer reaction, which occurs in the nucleus. Thus, retroviral integrase must recognize the specific viral DNA and integrate viral donor DNA to nonspecific target DNA substrates in the overall integration process. This raises the question whether retroviral integrase has one or two DNA binding sites and if each DNA binding requires divalent metal ions.

DNA binding studies of HIV-IN utilizing various techniques have been previously reported (Schauer & Billich, 1992; van Gent et al., 1991; Hazuda et al., 1994; Engelman et al., 1994). The results of these studies indicate that there is little or no sequence specificity for binding of HIV-1 integrase to DNA: single-stranded and duplex nonspecific DNA substrates can bind to integrase as efficiently as the U5 substrate and there is no correlation between the DNA binding and sequence-specific cleavage activity of HIV-1 integrase. It has been previously reported that the retroviral integrases of the Moloney murine leukemia virus (Roth et al., 1988), Rous sarcoma virus (Mumm & Grandgenett,

1991), and HIV-1 (Hazuda et al., 1994; Engelman et al., 1994), are capable of binding to DNA in the absence of divalent metal ions. In contrast, another report indicates that binding of HIV-1 integrase to DNA requires divalent metal ions (Schauer & Villich, 1992). It should be emphasized that the these previous studies have used short duplex oligonucleotides that correspond to the termini of HIV-1 DNA.

Our results indicate that these short oligonucleotide substrates have less DNA binding affinity as compared to the longer substrates for the 3′-processing activity. It has been previously reported that the 15-bp oligonucleotide is a substrate for specific cleavage and integration but not as good a substrate as the 28-bp oligonucleotide: about equal amounts of cleavage products of 14 nucleotides (a nonspecific product) and of 13 nucleotides (the specific product) were found when the 15-bp substrate was cleaved by IN (Vink et al., 1991). Furthermore, it has been previously shown that nonspecific DNA competes with a short oligonucleotide substrate in the presence of Mn^{2+} (Van Gent et al., 1991). Our results show that both short and long substrates can be utilized as target DNA in the presence of Mn^{2+}, indicating that HIV-IN has a nonspecific target DNA binding site. These results suggest that HIV-1 integrase has two DNA binding sites: a specific viral DNA binding site and a nonspecific target DNA binding site. This model has been previously suggested (Engelman et al., 1991; Vincent et al., 1993). The existence of two DNA binding sites in the integrase protein can explain why there is no sequence specificity for binding of HIV-1 integrase to DNA and why the observed DNA binding to HIV-IN does not correlate with the specific activity of the enzyme.

Functional Role of Divalent Cations. Divalent metal cofactor(s) may have a role in influencing the conformation of the DNA substrate and thereby enhancing the specificity of the DNA binding. Generally, nucleases require divalent metal cofactor(s) for catalysis and/or DNA binding. In the absence of divalent ions, restriction enzymes cannot cleave their respective substrates but they can bind to DNA (Terry et al., 1987; Aiken et al., 1991; Xu & Schildkraut, 1991; Gabbara & Bagwat, 1992; Zebala et al., 1992). In these cases, the metal ion is required for catalysis rather than DNA binding. In binding to DNA in the absence of metals, restriction enzymes show very different levels of specificity for their recognition sites. *Eco*RI binds more tightly to its recognition sequence than to noncognate DNA sequences (Halford & Johnson, 1980; Terry et al., 1983), whereas *Eco*RV has the same affinity for all DNA sequences and translocates readily from one site to another along the DNA (Taylor et al., 1991). However, when *Eco*RV is located at its recognition site, the enzyme has a high affinity for Mg^{2+} ions, but when located at a noncognate site, the enzyme has a low affinity for Mg^{2+} (Taylor & Halford, 1989). Therefore, conformational changes in proteins and/or DNA structures associated with interactions between proteins and DNA substrates can determine metal binding affinity (i.e., Mg^{2+}). In these cases, at least part of the specificity of the *Eco*RV stems from the way in which the affinity of the *Eco*RV−DNA complex for Mg^{2+} varies with the DNA sequence (Winkler et al., 1993).

It is envisioned that the affinity of metal binding in integrases may vary depending on the sequence of DNA, as with the *Eco*RV−DNA complex, or that the binding of a metal cofactor confers the proper protein conformation for the formation of tight complexes with DNA substrates. The results presented in these studies demonstrate that (1) the 35-bp DNA substrate displays a higher DNA binding affinity as compared to the 24-bp DNA substrate, (2) the DNA binding affinity of the longer substrate is slightly higher in the presence of Mg^{2+} than Mn^{2+}, and (3) the reactivity of shorter substrate requires a higher concentration of Mg^{2+} than the longer substrate. Therefore, length-dependent structure of DNA not only influences the specificity of DNA binding but also differentiates the optimal metal concentration for the activity of HIV-IN. Mg^{2+} may bind the integrase protein and subsequently induce the conformational changes of the protein, thereby affecting the specificity of the HIV-IN functions.

Conclusion. Functional studies of the viral integration process rely heavily on the efficacy of the *in vitro* assays. The results presented in this paper provide an opportunity to reevaluate the substrate requirements for the *in vitro* assay systems. Although short duplex oligonucleotides that correspond to the termini of HIV DNA can be used as substrates in the presence of Mn^{2+}, these short substrates are less efficient for the Mg^{2+}-dependent activity of HIV-IN. Our findings indicate that HIV-IN can catalyze the Mg^{2+}-dependent 3′-processing activity and may improve the current *in vitro* systems to be used for characterizing functions and eventually screening drugs for the control of specific steps of the viral integration process.

This study demonstrates that the structure of DNA associated with different length substrates has effects on both DNA and metal binding affinites. These structural changes resulted in differential effects on 3′-processing activity. Consequently, our studies have established the existence of a Mg^{2+}-dependent activity of wild-type HIV-IN *in vitro*. However, this Mg^{2+}-dependent activity does not efficiently promote the half-site autointegration. This is an indication that other accessory viral or host factors may play an important role in mediating the integration reaction *in vivo*. One observation indicating the participation of other factors is that endogenous viral DNA made by reverse transcription exists as part of a large nucleoprotein complex derived from the viral core. Integrase catalyzes the integration of this DNA as a specific subviral nucleoprotein complex but not as naked DNA (Brown et al., 1987; Bowerman et al., 1989). This is supported by the observation that efficiency increases in cell-free integration reactions with the addition of cytoplasmic extract (Fujiwara & Craigie, 1989). Although far from providing a complete understanding of the overall mechanism of viral integration, these functional studies of HIV-IN with various substrates have demonstrated that the longer substrates provide results more consistent with studies with endogenous viral DNA and infected cell extracts, thereby potentially reconciling the discrepancy between the *in vivo* and *in vitro* HIV-IN-mediated reactions. Further extensive *in vitro* characterizations may provide a better understanding of the overall mechanism of the viral integration process.

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