Zinc Stimulates Mg^{2+}-Dependent 3′-Processing Activity of Human Immunodeficiency Virus Type 1 Integrase *in Vitro*

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ABSTRACT: Human immunodeficiency virus type 1 integrase (HIV-1 IN) catalyzes both 3′-donor processing and strand transfer reactions. Previous studies have determined that the N-terminal region, a putative zinc finger, is capable of binding Zn^{2+}. The function of zinc coordination to this domain, however, is still unknown. In this report, we present evidence that Mg^{2+}-dependent 3′-donor processing by HIV-1 IN is enhanced by the addition of Zn^{2+} *in vitro.* This activity is inhibited in the presence of the chelator 1,10-phenanthroline (OP). In addition, the Mg^{2+}-dependent 3′-donor processing activity is more sensitive to the concentration of IN than is the Mn^{2+}-dependent activity. A combination of dimethyl sulfoxide (DMSO) and poly(ethylene glycol) (PEG) was found to further activate the Mg^{2+}-dependent 3′-donor processing activity while diminishing the Mn^{2+}-dependent activity. These results suggest factors such as substrate-length, concentration of IN, Zn^{2+} coordination, and protein−protein interactions are important for efficient and specific donor processing activity with Mg^{2+} *in vitro.*

The steps involved in retroviral integration are essential for retroviral replication and, thus, are attractive targets for inhibiting the life cycle of retroviruses. The integration process requires two distinct reactions: the sequence-specific cleavage of the 3′ ends of the linear double-stranded viral DNA termini (3′-donor processing) in the cytoplasm (Roth et al., 1989) and joining of the resulting recessed viral DNA into the host chromosomal DNA (strand transfer) in the nucleus. The strand transfer reaction results in a gapped recombinant intermediate DNA structure which presumably requires DNA repair enzymes to complete the integration process. *In vitro* studies have demonstrated that purified recombinant retroviral integrases are capable of catalyzing the 3′-processing and strand transfer reactions with short oligonucleotide substrates (Katzman et al., 1989; Craigie et al., 1990). In addition, two other reactions have been observed *in vitro*: disintegration (Chow et al., 1992) and alternative disintegration (Lee et al., 1995a).

The integrases of retroviruses and other retrotransposases (e.g., Ty 1) share several highly conserved amino acid residues and motifs (Johnson et al., 1986; Katzman et al., 1991; Khan et al., 1991). Amino acid sequence comparisons of retroviral integrases (INs)[1] and mutational analyses have identified three distinct functional regions in the IN proteins: a N-terminal region (residues 1−50), a central core domain (residues 60−160), and a C-terminal domain (residues 200−270). The N-terminal domain contains a HHCC region, a putative zinc-finger motif (Johnson et al., 1986). It has been reported that mutant proteins containing substitutions of these His or Cys residues are more defective for 3′ processing and strand transfer than for disintegration activity (Engelman & Craigie, 1992; van Gent et al., 1992; Leavitt et al., 1993; Vincent et al., 1993). Accordingly, this domain seems to be required for both the donor processing and the strand transfer reactions although its exact function remains unknown. The central core region contains three acidic residues (Asp64, Asp116, and Glu152), the "D,D-X35-E" motif (Drelich et al., 1992; Kulkosky et al., 1992). This motif is conserved in the transposases of certain bacterial transposons (e.g., MuA, Tn3, Tn7), and mutations of these acidic residues lead to the inactivation of IN, suggesting that they are essential for catalysis of polynucleotidyl transfer (Drelich et al., 1992; Engelman & Craigie, 1992; van Gent et al., 1992; Kulkosky et al., 1992; Vincent et al., 1993). These acidic amino acids have been proposed to bind divalent cations. Indeed, the catalytic functions of IN are not observed in the absence of divalent cations. The C-terminal region is the least conserved region of the retroviral integrases. It has been determined that this domain is the major nonspecific binding determinant of IN (Khan et al., 1991; Woerner & Marcus-Sekura, 1993; Vink et al., 1993; Engelman et al., 1994). Since HIV-1 IN has to bind both the donor viral DNA (3′-processing) and the host target DNA (strand transfer), it is reasonable that HIV-1 IN has been shown to have only a slightly higher affinity for specific viral DNA than for nonspecific DNA (van Gent et al., 1991).

Complementation studies with IN proteins mutated in different domains indicate that both the N- and C-termini are required for site-specific cleavage and strand transfer reactions, although they are not essential for the disintegration reaction (Vink et al., 1993; Engelman et al., 1993). Furthermore, these studies determined that certain mixtures of individually inactive mutant proteins are active for both 3′-processing and strand transfer mixed. These *in vitro* complementation experiments suggest that the active form of integrase is an oligomer, although they do not determine the precise functional unit (e.g., dimer vs tetramer).

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[1] Abbreviations: bp, base pair(s); CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PEG, poly(ethylene glycol) 8000; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HIV-1, human immunodeficiency virus type 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT, His-tag; IN, integrase protein (integrase); LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; OP, 1,10-phenanthroline; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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Recent mutational studies have shown that the single amino acid substitution of Phe185 to Lys increases the solubility of the central core domain (Dyda et al., 1994; Jenkins et al., 1995). The improved solubility allowed for this mutant central core domain to be crystallized and the X-ray crystal structure to be resolved (Dyda et al., 1994). These studies determined that the mutant core domain was in fact in the form of a dimer. In addition, the folding pattern of the crystal structure was strikingly similar to the core domain of bacteriophage MuA, a transposase which also contains the three essential acidic amino acids [see Dyda et al. (1994) and references cited therein]. The well characterized MuA utilizes either Mg^{2+} or Mn^{2+} for its activities *in vitro* (Baker et al., 1991). Similarly, reactions with purified IN and short DNA substrates matching the ends of the viral DNA can utilize either divalent cation. Several reports have shown that Mg^{2+} can efficiently support the 3′-processing activities (Vink et al., 1991; Drelich et al., 1992; Vincent et al., 1993; Bushman & Wang, 1994; Lee et al., 1995a,b; Engelman & Craigie, 1995) and strand transfer activities (Bushman & Wang, 1994; Engelman & Craigie, 1995; Vora et al., 1994) of retroviral INs *in vitro*. These *in vitro* reactions are consistent with results from Mo-MLV viral DNA−protein complexes isolated from infected cell extracts which utilize either Mg^{2+} or Mn^{2+} (Brown et al., 1987; Fujiwara & Mizuuchi, 1988).

Our recent studies have demonstrated that recombinant HIV-1 IN effectively utilizes Mg^{2+} for efficient 3′-donor processing *in vitro* and that Mg^{2+} is the preferred divalent cation with a longer donor DNA substrate: HIV-1 IN removes two nucleotides at the 3′-end of the 35 bp oligonucleotide substrate in the presence of Mg^{2+} while the shorter 24 bp oligonucleotide substrate was cleaved more efficiently in the presence of Mn^{2+} than Mg^{2+} (Lee et al., 1995b). Substrate competition studies indicated that a nonspecific DNA substrate was not able to compete with the longer substrate (35 bp DNA), whereas the shorter substrate (24 bp DNA) was easily competed off by the nonspecific DNA substrate. These results suggest that the IN protein has a higher affinity for the longer oligonucleotide substrate.

In this report, HIV-1 integrase-mediated 3′-donor processing and strand transfer reactions were further characterized under various reactions conditions. DMSO and poly(ethylene glycol) (PEG) slightly increased the Mg^{2+}-dependent 3′-processing and strand transfer reactions, while reducing the Mn^{2+}-dependent activities significantly. In addition, we have observed that the addition of Zn^{2+} stimulated the Mg^{2+}-dependent 3′-processing activity, while 1,10-phenanthroline (OP) totally inhibited the Mg^{2+}-dependent 3′-processing activity. In contrast, Mn^{2+}-dependent activities of HIV-1 IN were much less sensitive to the addition of Zn^{2+} or OP. The results presented in these studies provide new insights on the structure/function relationship of HIV-1 IN.

MATERIALS AND METHODS

Purification of the IN Proteins. Both wild-type and "His-tag" IN proteins were overexpressed in *Escherichia coli*, BL21(DE3), and the IN proteins were purified according to the previously described procedure (Sherman & Fyfe, 1990). Both plasmids containing the gene encoding HIV-1 integrase were obtained from Dr. R. Craigie.

Oligonucleotide Substrates. Oligonucleotides were purchased from Integrated DNA Technologies Inc. The oligonucleotides were purified by gel electrophoresis through 7 M urea 20% denaturing gels. The appropriate bands were cut by UV shadowing and electroeluted using the S&S Elutrap electroseparation system from Schleicher & Schuell. The DNA was then ethanol-precipitated. The substrate utilized in this study corresponds to the terminal 35 bp of the U5 LTR end:

5′-**GACCCTTTTAGTCAGTGTGGAAAATCTCT-AGCAGT**

3′-**CTGGGAAAATCAGTCACACCTTTTAGAGA-TCGTCA**

Radioactive DNA Cleavage and Strand Transfer Reactions. One microgram of the appropriate oligonucleotide was ^{32}P-labeled at the 5′ termini by use of T4 polynucleotide kinase and 25 μCi of adenosine 5′-[γ-^{32}P]triphosphate. The labeled oligonucleotide was annealed with a 3-fold molar excess of unlabeled complementary strand in 10 mM Tris-HCl, pH 8.0, 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), 7.5 mM $MgCl_2$ or $MnCl_2$, 0.5 pmol of ^{32}P-labeled substrates, and 0.4 μM wild-type HIV-1 IN (unless otherwise stated) in a total volume of 20 μL. The reactions were initiated by the addition of IN protein, and the reaction mixtures were incubated up to 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 boiled for 5 min. Ten microliters of each reaction mixture was electrophoresed on a 7 M urea denaturing 18% polyacrylamide sequencing gel, and reaction products were analyzed by autoradiography. Quantitation of 3′-processing activity was performed with a Pdi densitometer Model DNA 35 with a Kodak photographic step tablet for the calibration curve. Zinc chelation experiments were performed with 1,10-phenanthroline (Aldrich Chemical Co.) under the standard reaction conditions.

RESULTS

Zn^{2+} Stimulates the Mg^{2+}-Dependent 3′-Processing Activity of HIV-1 IN. The N-termini of retroviral integrases contain highly conserved amino acid residues. It has been previously postulated that the HHCC region, containing two histidine and two cysteine residues, could be a putative zinc-finger domain (Johnson et al., 1986). In general, substitutions of these residues result in proteins that are defective in their 3′-processing and strand transfer activity, although they somewhat retain their disintegration activity (Khan et al., 1991; Engelman & Craigie, 1992; van Gent et al., 1992; Leavitt et al., 1993; Vincent et al., 1993). The functional significance of this region remains unclear. To address this question, the effects of Zn^{2+} on HIV-1 IN-mediated reactions were characterized with both wild-type and "His-tag" IN (HT-IN) proteins. The activities of HIV-1 IN were analyzed in the presence and absence of 10 μM $ZnSO_4$ in 7.5 mM $MgCl_2$ or 7.5 mM $MnCl_2$.

Figure 1 represents the *in vitro* 3′-processing and strand transfer assays performed with four different preparations of HT-IN (Figure 1A) and three different preparations of wild-type IN (Figure 1B). Both wild-type IN and HT-IN were purified using identical purification procedures which require ammonium sulfate precipitation and two column

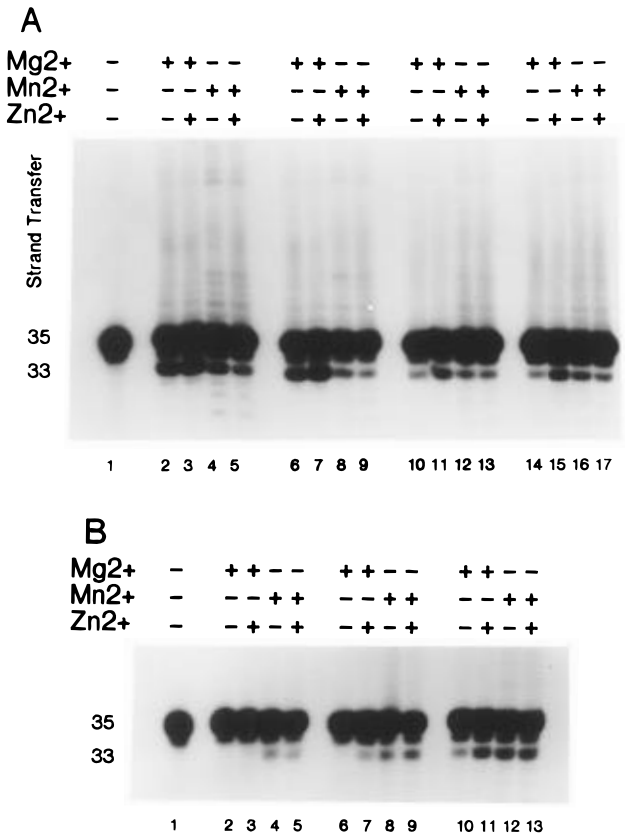

FIGURE 1: 3′-Processing and strand transfer analysis of different HIV-1 integrase preparations. Reactions were performed as described under Materials and Methods. For each integrase preparation, 7.5 mM $MgCl_2$ or 7.5 mM $MnCl_2$ was compared in the absence and presence of 10 μM $ZnSO_4$. The various combinations are indicated on the figure as + or −. Reactions with (A) "His-tag" integrase and (B) wild-type integrase. Lanes 1 in panels A and B represent the substrate in the absence of integrase. In (A), lanes 2−5 represent reactions in which the concentration of IN used was 420 nM; lanes 6−9, 300 nM; lanes 10−13, 194 nM; and lanes 14−17, 160 nM. In (B), lanes 2−5 represent reactions in which the concentration of integrase used was 46 nM; lanes 6−9, 93 nM; and lanes 10−13, 120 nM. Each reaction contained 0.5 pmol of 35 bp U5 LTR in a 20 μL reaction volume. Products were separated by a 7 M urea denaturing 18% polyacrylamide gel and analyzed by autoradiography.

chromatographic steps: butyl-Sepharose and heparin−Sepharose columns (Sherman & Fyfe, 1990). All of the IN proteins were dialyzed against 25 mM HEPES, pH 7.5, 1 mM EDTA, pH 8.0, 1 mM DTT, 1 M NaCl, and 20% glycerol (w/v) in the final step. These proteins were frozen in liquid nitrogen and stored at −80 °C. The only variation among the different preparations was their final protein concentrations. Functional assays were performed by the addition of 1 μL of each protein sample to a 20 μL total reaction volume, resulting in a final NaCl concentration of 50 mM. The autoradiograph of the gel data is shown in Figure 1, and the quantitated data analyzed by a densitometer are summarized in Table 1.

The results from Figure 1 provide three important features: (1) When the 3′-processing activities with Mg^{2+} were compared to those with Mn^{2+}, significant variations in the efficiency of DNA cleavage were noticed with different preparations of the IN protein. Evidently, the reactions with the IN protein whose stock concentrations were low resulted in more efficient cleavage with Mn^{2+} than Mg^{2+} (Figure 1B). At protein concentrations lower than 200 nM, the Mn^{2+}-activated cleavage produced higher amounts of processed DNA than the Mg^{2+}-dependent reactions. In contrast, the efficiency of the Mg^{2+}-dependent cleavage activity increased with higher concentrations of IN assayed. In these reactions, more products were formed with Mg^{2+} than with Mn^{2+} (Figure 1A). The results from the wild-type IN and HT-IN proteins were similar. Therefore, there are no apparent variations in the activity of IN due to the presence of the "His-tag" under these conditions. In fact, concentration-dependent activation of endonuclease activity has been previously noted for both the HIV-1 (Engelman & Craigie, 1992) and RSV (Jones et al., 1992) integrases. This effect has been ascribed to the necessity of multimerization. Accordingly, one explanation for the results observed in Figure 1 could be that protein−protein interactions are important for the Mg^{2+}-dependent activity of HIV-1 IN. (2) The addition of Zn^{2+} resulted in activation of cleavage reactions predominantly with Mg^{2+}. This activation was not observed from reactions with Mn^{2+}. Therefore, it seems that the addition of exogenous Zn^{2+} is important for the Mg^{2+}-dependent reaction while not affecting assays performed in the presence of Mn^{2+}. (3) The degree of activation as a result of Zn^{2+} addition was more significant with low IN concentrations, whereas reactions with higher protein concentrations were not dramatically enhanced by the addition of Zn^{2+}. For example, the reactions with 194 nM HT-IN are interesting (Figure 1A, lanes 10−13). In the presence of only Mn^{2+}, 5.8% of the substrate was cleaved (lane 12) while 4.6% cleavage products were observed from the reaction with Mg^{2+} (lane 10). However, the addition of 10 μM Zn^{2+} resulted in conversion of 12.3% of the substrate with Mg^{2+} (lane 11) while it resulted in formation of 5.3% cleavage products with Mn^{2+} (lane 13). Thus, the addition of Zn^{2+} resulted in nearly 3-fold activation in the reaction with Mg^{2+} while the Mn^{2+}-dependent reactions did not significantly change. More dramatic results were observed from reactions with 93 nM wild-type IN (Figure 1B, lanes 6−9). In the presence of Mg^{2+}, significant amounts of products were not observed (lane 6), while 5.8% of the substrate was cleaved with the addition of Zn^{2+} (lane 7). In contrast, 14% cleavage of substrate with Mn^{2+} (lane 8) was reduced to 11.3% cleavage in the presence of Zn^{2+}. Hence, once again both the wild-type IN and HT-IN proteins showed similar zinc effects, indicating that the presence of the "His-tag" did not significantly affect the activity of IN under these conditions.

Table 1: Effects of Zn^{2+} Coordination on the 3′-Processing Reaction[a]

prepn	lanes	protein concn assayed (nM)	% cleavage			
			Mg^{2+}	Mg^{2+}/Zn^{2+}	Mn^{2+}	Mn^{2+}/Zn^{2+}
"His-tag"	2−5	420	21.5	22.5	12.7	9.7
	6−7	300	17.5	24.6	8.2	5.3
	10−13	194	4.6	12.3	5.8	5.3
	14−17	160	3.8	11.3	7.2	5.2
wild type	2−5	46	0.7	1.0	5.6	4.7
	6−9	93	1.3	5.8	14.0	11.3
	10−13	120	6.2	14.9	16.2	14.8

[a] Reactions were performed as described in the text. Percent cleavage was determined with a Pdi densitometer Model DNA 35 with a Kodak photographic step tablet for the calibration curve.

Characterization of Different Divalent Cations. To confirm that the enhanced activity observed by the addition of exogenous Zn^{2+} is not due to the ability of Zn^{2+} to catalyze

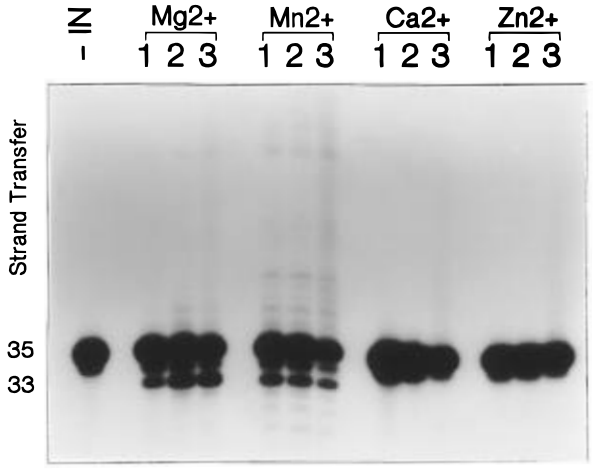

FIGURE 2: Characterization of different divalent cations on the 3′-processing and strand transfer reactions of HIV-1 IN. The divalent cations used are indicated on the figure. −IN represents the substrate alone; the integrase has been omitted. Lanes 1−3 represent reactions in which the final concentrations of the divalent cation are 1, 2.5, and 7.5 mM, respectively. Products are indicated on the left by the 33 Size Marker and Strand Transfer.

the 3′-processing reaction, different divalent cations were examined for their 3′-processing and strand transfer ability. Reactions in the presence of 1, 2.5, or 7.5 mM of either Mg^{2+}, Mn^{2+}, Zn^{2+}, or Ca^{2+} were analyzed with wild-type HIV-1 IN. As shown in Figure 2, Mg^{2+} and Mn^{2+} resulted in cleavage products for all concentrations of the divalent cation. In contrast, both Ca^{2+} and Zn^{2+} did not catalyze the 3′-processing reaction. Therefore, these divalent cations (Ca^{2+} and Zn^{2+}) do not independently catalyze 3′-processing. This indicates that the results seen in Figure 1 are not the result of Zn^{2+} coordinating into the "D,D,X35-E" motif. Hence, the enhancement seen in Figure 1 was the result of a different mechanism. We hypothesized that the Zn^{2+} was important in allowing for protein−protein interactions to occur through coordination into the putative zinc finger. To examine this possibility, the effects of OP on 3′-processing activity were further examined.

Effects of OP on the Activities of HIV-1 IN. Since integrase requires divalent cations as metal cofactors (either Mg^{2+} or Mn^{2+}), it is difficult to examine the effects of Zn^{2+} removal by EDTA. EDTA has K_1 stability constants of 9, 14, and 16 toward Mg^{2+}, Mn^{2+}, and Zn^{2+}, respectively (Auld, 1988). One approach to selectively remove Zn^{2+} from the protein is to use more specific chelators such as the phenanthrolines. 1,10-Phenanthroline (OP) forms only very weak complexes with Mg^{2+}, but binds much higher to Mn^{2+} or Zn^{2+} (β_3 of OP is 7 and 17.6 for Mn^{2+} and Zn^{2+}, respectively) (Auld, 1988). Hence, for enzymes that are activated by Mg^{2+} or Mn^{2+}, it is more reasonable to select Mg^{2+} as the activating cation and OP as the zinc chelating agent to investigate the possible requirement of Zn^{2+} for the activity of the integrase enzyme.

Figure 3A represents the 3′-processing activity of HIV-1 integrase as a function of the concentration of OP in the presence of Mg^{2+} or Mn^{2+}. The protein concentration assayed was 0.4 μM. For Mg^{2+}-dependent reactions, integrase became inactive when the concentration of OP was higher than 20 μM (Figure 3A). When the same experiments were repeated in the presence of 10 μM Zn^{2+}, the OP-induced inactivation was not observed (Figure 3B) although higher

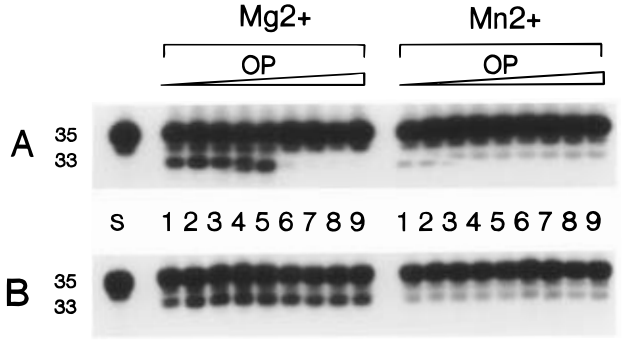

FIGURE 3: Effects of 1,10-phenanthroline on the 3′-processing reaction in the absence and presence of Zn^{2+}. (A) Reactions performed in the presence of 7.5 mM $MgCl_2$ or 7.5 mM $MnCl_2$. (B) Reactions performed in the presence of 10 μM $ZnSO_4$. Lanes 1−9 represent increasing concentrations of OP: 0, 1, 5, 10, 15, 20, 30, 40, and 50 μM OP, respectively.

concentrations of OP (>100 μM) did inhibit the activity (data not shown). This failure to inhibit activity at 50 μM in the presence of 10 μM Zn^{2+} can be attributed to the fact that OP forms a 2:1 complex for Zn^{2+} binding. Second, OP forms mono-, di-, and tridentate species depending on the concentration of OP. Therefore, the metal can form mixed complexes with different species of OP. The di- and tridentate species form stronger complexes with metal than the monodentate species. The concentration of OP which will effectively compete the apoenzyme for zinc will be in the concentration range in which the di- and tridentate species are favored. However, the ratio of these species may not necessarily be altered linearly with increasing concentrations of OP. In fact, a 10-fold increase in chelator concentration from 1 μM to 10 μM can change the ratio of di- plus tri- to monodentate species by 184-fold (Auld, 1988). Hence, it is conceivable that the effectiveness of metal chelation and subsequent inhibition of enzymatic activity may not be linear with changes in OP concentration. This would explain why the addition of 10 μM Zn^{2+} prevents inactivation by 50 μM OP. In addition, the sharp decrease in activity from 15 to 20 μM OP can also be attributed to this line of reasoning.

In contrast, the Mn^{2+}-dependent 3′-processing activity was not altered by the addition of OP up to 50 μM. It appears that OP preferentially chelated Zn^{2+} in the presence of Mg^{2+}, but not in the presence of Mn^{2+}. This may be explained by the different metal binding affinities of OP toward the three different divalent cations. Although Zn^{2+} binds to OP tighter than does Mn^{2+}, the difference in the affinity is not large. Therefore, at lower concentrations of OP, Mn^{2+} will compete with the Zn^{2+} for binding to OP, resulting in a lack of apparent inhibition.

Hence, the Zn^{2+} chelation experiments were repeated with higher concentrations of OP (Figure 4). The Mn^{2+}-dependent activity was inhibited at a higher concentration of OP (1 mM) than the Mg^{2+}-dependent activity. Both the Mg^{2+}- and Mn^{2+}-dependent 3′-processing activities of IN were inhibited at 1 mM OP. The inhibition at this concentration of OP (1 mM) cannot account for chelation of either the Mg^{2+} or the Mn^{2+} cations, which were 7.5 mM. As observed in Figure 2, even 1 mM cation results in activity. These results argue for the requirement of zinc coordination to a putative zinc binding domain.

The aforementioned results collectively suggest that there are apparent protein concentration- and Zn^{2+}-dependent

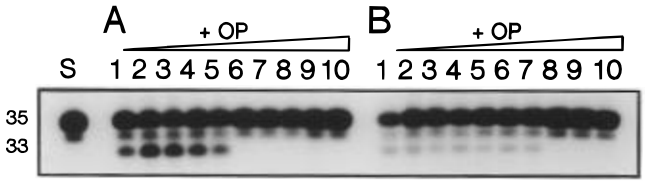

FIGURE 4: Effects of 1,10-phenanthroline on the 3′-processing reaction. (A) Reactions performed in 7.5 mM $MgCl_2$. (B) Reactions performed with 7.5 mM $MnCl_2$. (S) Substrate in the absence of integrase. Lanes 1−10 employed 0, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 1 mM, 5 mM, and 10 mM OP, respectively.

activities. It seems that Zn^{2+} promotes an apparent protein−protein interaction which could be essential for the specific function of HIV-1 IN. Such Zn^{2+}-dependent protein−protein interactions preferentially activate the Mg^{2+}-dependent activity as compared to the Mn^{2+}-dependent activity. It has been previously suggested that Zn^{2+} coordination to the putative zinc-finger domain may not be required for the activities of the HIV-1 IN proteins (Leavitt et al., 1993). This conclusion was drawn from activity assays performed exclusively with Mn^{2+} (Leavitt et al., 1993). Our results also support this previous conclusion in the presence of Mn^{2+}. However, our results indicate that this conclusion is not consistent with the Mg^{2+}-dependent 3′-processing activities of HIV-1 IN. Based on the results presented in this study, we propose that Zn^{2+} coordination to a putative zinc-finger domain induces conformational changes in the IN protein which may promote protein−protein interactions required for Mg^{2+}-dependent 3′-processing.

Effect of Various Reaction Conditions on the 3′-Processing Activity of HIV-1 IN. It has been previously shown that the *in vitro* activities of HIV-1 IN are sensitive to changes in pH, salt concentration, protein concentration, and concentration of divalent cations (Vincent et al., 1993; Lee et al., 1995a,b). We examined the effects of various reagents in the reaction conditions on both 3′-processing and strand transfer activities of HIV-1 IN. These reagents include glycerol, NP-40 (nonionic detergent), CHAPS (zwitterionic detergent), an organic solvent (DMSO), and poly(ethylene glycol) (PEG). For these experiments, the standard reaction buffer contained 0.5 pmol of radiolabeled 35 bp DNA substrate, 0.4 μM HIV-1 wild-type IN, 25 mM HEPES, pH 7.5, 1 mM DTT, 50 mM NaCl, and either 7.5 mM $MgCl_2$ or 7.5 mM $MnCl_2$ in a total of 20 μL. The autoradiograph of the gel is shown in Figure 5, and the data quantitated by a densitometer are summarized in Table 2.

There are four intriguing observations from Figure 5: (1) The donor 3′-processing reaction of HIV-1 IN was more efficient with Mg^{2+} than Mn^{2+} under all of the reaction conditions tested. This is consistent with previous results that showed that the 35 bp U5 DNA substrate was more efficiently cleaved in the presence of Mg^{2+} than Mn^{2+} (Lee et al., 1995a,b). (2) Some of these reagents show differential effects on the Mg^{2+}-dependent activities as compared to the Mn^{2+}-dependent activity. Glycerol, CHAPS, and NP-40 show marginal effects on both Mg^{2+}- and Mn^{2+}-dependent activities. However, DMSO and PEG each slightly increased the efficiency of the Mg^{2+}-dependent 3′-processing and strand transfer activities while significantly reducing these activities in the presence of Mn^{2+}. (3) The most dramatic differences were observed when all of the reagents were combined together (Figure 5B, lane k): the 3′-processing activity in the presence of Mg^{2+} was increased by 30% as

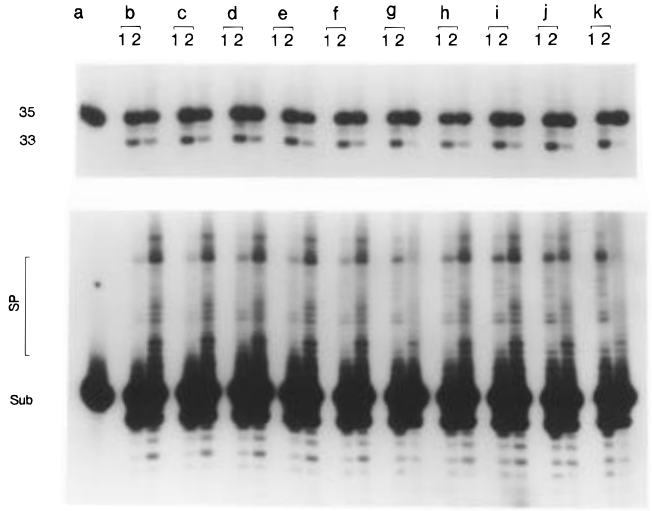

FIGURE 5: Effects of different reaction conditions on the 3′-processing and strand transfer reactions. Top panel, 3′-processing reaction products. Bottom panel, strand transfer products (SP). Reaction conditions in lanes b−k correspond to the reaction conditions described for the respective letters in Table 2. In each set, lanes 1 and 2 represent reaction conditions in the presence of 7.5 mM $MgCl_2$ and 7.5 mM $MnCl_2$, respectively. (a) Reaction where integrase has been ommitted. Strand transfer products were observed by overexposing the same gel.

compared to an 18-fold reduction in the presence of Mn^{2+}. In addition, this combination of DMSO and PEG significantly enhanced the strand transfer activity much more than the increase in 3′-processing activity in the presence of Mg^{2+}. (4) The patterns of strand transfer products with Mg^{2+} were not identical to those with Mn^{2+}. As seen in Figure 5, the preferred sites of integration are distinctively different depending on the divalent cation.

The effects of DMSO and PEG are especially interesting. PEG increases macromolecular interactions through volume exclusion; DMSO alters the hydrophobicity of proteins by lowering the dielectric constant of the solvent. Therefore, one hypothesis is that these reagents enhance the Mg^{2+}-dependent reaction by increasing protein−protein interactions and/or the stability of the protein. The interactions of divalent cations in the core domain or the N-terminal domain of HIV-1 IN may be important for maintaining the appropriate conformation to allow for protein−protein interactions and/or for the stability of the protein. Since metals frequently are involved in structural as well as functional roles, the stability of the apoprotein is also a particularly important consideration.

Characterization of the Effects of DMSO and PEG in the Presence of OP. The aforementioned data showed the activation of the Mg^{2+}-dependent activities of integrase either by the addition of Zn^{2+} or by a combination of DMSO and PEG. One consideration in the data interpretation of these results is that the enhancement by DMSO and PEG may be due to adventitious Zn^{2+} contamination in these reagents. Since the enzyme is stored in the presence of 1 mM EDTA, a fraction of the enzyme mixture may be in the form of apoproteins. Hence, the enhanced activity observed with DMSO and PEG could be the result of Zn^{2+} contamination rather than to the solution-modifying properties.

To further examine whether the effects of DMSO and PEG are independent of zinc coordination, experiments were performed in the presence of OP. The results are shown in

Table 2: Effects of Various Reaction Conditions on the 3′-Processing Activity of HIV-1 IN[a]

lanes	% cleavage with Mg^{2+}	% cleavage with Mn^{2+}	glycerol concn (%)	CHAPS concn (mM)	NP-40 concn (%)	DMSO concn (%)	PEG concn (%)
b	24	16.6	10				
c	25.9	13.4	10	0.5			
d	23.3	17.3	10		0.05		
e	27.6	11.5	10	0.5	0.05		
f	25.9	13.6		0.5	0.05		5
g	26.6	6.3		0.5	0.05		10
h	26.7	9.3	10			10	
i	27.5	10.5	10	0.5	0.05	10	
j	31.9	8.7	10	0.5	0.05	10	5
k	30.8	0.9	10	0.5	0.05	10	10

[a] Reactions were performed as described in the text.

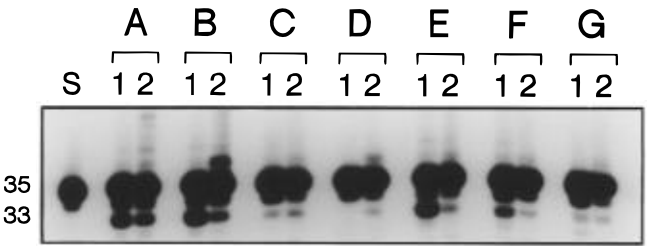

FIGURE 6: Effects of 1,10-phenanthroline on 3′-processing in the presence of PEG and DMSO. All reaction mixtures contained final concentrations of 25 mM HEPES, pH 7.5, 10% glycerol, 5 mM DTT, 0.05% NP-40, 0.5 mM CHAPS, and 50 mM NaCl. (A) Reactions where the only addition was the divalent cation. (B) Reactions in the presence of 10 μM $ZnSO_4$. (C) Reactions in the presence of 100 μM OP. (D) Reaction in the presence of 200 μM OP. (E) Reaction in the presence of DMSO and PEG. (F) Reaction with DMSO and PEG in the presence of 100 μM OP. (G) Reaction with DMSO and PEG in the presence of 200 μM OP. S represents the substrate where integrase has been omitted. Lanes 1 and 2 in each set are reaction with 7.5 mM $MgCl_2$ and 7.5 mM $MnCl_2$, respectively.

Figure 6. The presence of DMSO and PEG required a higher concentration of OP to inhibit the Mg^{2+}-dependent 3′-processing activity. The presence of 100 μM OP reduced 3′-processing activity significantly, but not completely in the presence of DMSO and PEG. Therefore, another hypothesis could be that these reagents induce protein−protein interactions resulting in multimeric integrase, which as a result is more resistant to Zn^{2+} chelation by OP. In contrast, the combination of DMSO and PEG significantly inhibited the Mn^{2+}-dependent activities, but the presence of OP did not further abolish this activity. Although it is difficult to draw a definite conclusion, it seems that a combination of DMSO and PEG may promote apparent conformational changes in IN proteins, thereby activating Mg^{2+}-dependent activity while significantly reducing the Mn^{2+}-dependent activity.

DISCUSSION

Protein−DNA interactions are important for biological processes such as replication, transcription, integration, and DNA repair. The initiation, regulation, and termination of these processes are only possible in the presence of specific DNA-binding proteins. Presently, X-ray and NMR solution structures have been reported for numerous protein−DNA complexes and DNA-binding proteins. One important observation from these structural studies and sequence comparisons is that many of the DNA-binding proteins can be grouped into classes or families that use related structural motifs for the recognition of specific sequences of DNA. Some examples of these specific families of structural motifs are the helix−turn−helix, the helix−loop−helix, the leucine-zipper, and the zinc-finger proteins (Berg, 1989).

The N-terminus of retroviral integrases is characterized by the highly conserved HHCC region. This region contains two histidine and two cysteine residues and has been postulated to be a zinc-finger domain (Johnson et al., 1986). The HHCC region of retroviral integrases differs from that of the prototypical DNA-binding zinc fingers of TFIIIA and in the nucleocapsid protein of retroviruses (South et al., 1990). The N-terminal domain of transcription factor IIIA (TFIIIA) contains 9 tandem repeats of a 30-residue domain, with each domain containing the sequence pattern Cys-X_{2or4}-Cys-X_{12}-His-X_{3-5}-His (Miller et al., 1985). The two cysteines, which are near a turn in the β-sheet region, and two histidines, in the α-helix, coordinate a zinc ion that holds these secondary structures together to form a compact globular domain. In contrast, the number of amino acid residues between the HH and the CC region in the IN proteins is about twice that of TFIIIA and much more than that in the nucleocapsid protein. The order of the conserved residues, HHCC, is also different from that found in any of the characterized zinc fingers in that the histidines come before the cysteines in the primary sequence. Nevertheless, the HHCC region has been predicted to coordinate zinc binding similar to that formed by the CCHH motif, since the formation of a zinc finger depends on the three-dimensional arrangement of the four coordinating residues rather than on their order in the primary sequence.

Experimental analyses of this region provide greater evidence that the HHCC region of HIV-1 IN coordinates zinc. Both direct spectroscopic studies with a synthetic 55 amino acid peptide (Burke et al., 1992) and Zn^{2+} blotting studies with various point mutant IN proteins (Bushman et al., 1993) have been conducted. The results from these mutational studies demonstrate that the two His and two Cys residues are indeed required for zinc binding. Point mutations of H12Q, C40S, and C43S resulted in the failure to coordinate a zinc ion. However, conflicting results arise in terms of the requirement of this zinc coordination for the overall activity of IN *in vitro*. Mutational studies in the HHCC region indicate that two point mutants of avian IN (H9N and H13N) and HIV-1 IN mutants (H16C and H16V) were active with Mn^{2+} without altering the pattern of processing of the LTR sequences (Khan et al., 1991; Leavitt et al., 1993). The data presented in the current studies are consistent with these previous observations. While Mn^{2+}-dependent 3′-processing activities showed no significant response to the addition of Zn^{2+} or a low concentration of

OP, the Mg^{2+}-dependent activities were drastically altered under the same conditions.

It is apparent that HIV-1 IN displays concentration-dependent activities. When a higher concentration of IN was assayed, the efficiency of 3′-processing was higher with Mg^{2+} than with Mn^{2+}. In contrast, the Mn^{2+}-activated cleavage reaction was superior to that of Mg^{2+} when the assayed protein concentration was low. Interestingly, the activation of the cleavage reaction by the addition of Zn^{2+} was also dependent on the IN concentration. Only slight increases in activation by zinc were observed with higher concentrations of IN (420 nM), whereas significant increases in activity were observed from cleavage reactions with lower IN concentrations. These increases in HIV-1 IN activities, however, were not observed from the reactions performed in the presence of Mn^{2+}. A comparison of the activities of HIV-1 IN in the presence of either Mg^{2+} or Mn^{2+} shows that there are many differences in activity: (1) At lower divalent cation concentrations, Mn^{2+} catalyzes the *in vitro* activities more efficiently than Mg^{2+} (Lee et al., 1995b). (2) Shorter DNA substrates are preferentially cleaved in the presence of Mn^{2+} compared to Mg^{2+}, whereas longer DNA substrates are more efficiently cleaved in the presence of Mg^{2+} (Lee et al., 1995b). (3) Substrate competition studies indicate that IN binds with a higher affinity to the 35 bp DNA substrate than to the 24 bp DNA substrate (Lee et al., 1995b). Furthermore, nonspecific DNA does not compete with the 35 bp substrate, whereas it does compete with the 24 bp substrate. (4) Mn^{2+} promotes efficient *in vitro* half-site strand transfer reactions utilizing the donor substrate as the target DNA, whereas Mg^{2+} significantly reduces the autointegration reactions (Lee et al., 1995b). Furthermore, Mg^{2+} supports concerted strand transfer reactions, whereas Mn^{2+} supports half-site strand transfer reactions (Vora et al., 1994). (5) Compared to Mg^{2+}, Mn^{2+} is more efficient at lower protein concentrations, whereas the opposite results are observed at higher IN concentrations (this study). (6) Mn^{2+} can support the disintegration reaction even with mutant IN proteins which are inactive in the donor processing and strand transfer reactions (Lee et al., unpublished results). Mg^{2+} does not promote efficient disintegration activity (Lee et al., 1995a). These results collectively indicate that Mn^{2+} may not be the appropriate metal cofactor for specific HIV-1 IN activity.

Differential activations of HIV-1 IN were also observed when the reactions were performed in the presence of DMSO and PEG. Generally, a combination of DMSO and PEG augmented the activity of HIV-1 IN with Mg^{2+}, while the activity of IN in the presence of Mn^{2+} was significantly decreased. Similar to the Zn^{2+} effect, DMSO and PEG increased the efficiency of 3′-processing when the reaction was performed with a low concentration of IN with Mg^{2+}. When the reaction was assayed with a high concentration of IN, the degree of activation in the cleavage reaction was similar to the zinc effect. The main difference observed with these effects was that the combination of DMSO and PEG resulted in an 18-fold reduction in the cleavage efficiency with Mn^{2+}, while the addition of Zn^{2+} caused only a slight reduction.

One explanation of these results is that the purified IN proteins are partial apoproteins, since the protein has been purified and stored in the presence of EDTA. This is further substantiated by the activation and inactivation observed in the presence of zinc and OP, respectively. It is also possible that the presence of adventitious zinc ions in the DMSO and PEG could result in rebinding of Zn^{2+} to the enzyme, thus resulting in the observed activation. In fact, IN was inactive in the presence of DMSO and PEG when the reaction was performed in the presence of OP. Although these results do not confirm whether the effects of Zn^{2+} and the combination of DMSO and PEG are related, it is clear that Zn^{2+} can stimulate the activities of HIV-1 IN in the presence of Mg^{2+}.

Interestingly, Mg^{2+} and Zn^{2+} are also essential for the activity of the large T antigens of SV-40 and polyomavirus. The large tumor antigen (LT) of the polyomavirus contains a CCHH zinc binding domain. Point mutations of the conserved CCHH residues inhibited the ability of the LT to function in viral DNA replication (Rose & Schaffhausen, 1995). Experiments with the large T antigen of SV40 have demonstrated that in the absence of magnesium, the large T antigen binds to the ends of linear DNA as a monomer or dimer (Schiedner et al., 1990). However, in the presence of magnesium, the large T antigen appeared to be assembled into oligomers. In addition, Mg^{2+} induced DNA looping behavior suggests that Mg^{2+}-dependent T antigen oligomerization is essential to the activities of SV40. Recent studies have shown that the N-terminal domain of HIV-1 IN is important for assembly of active multimers (Ellison et al., 1995). Such multimer assembly was not observed when the enzyme was treated with the thiol-modifying agent *N*-ethylmaleimide. Hence, it is conceivable that the role of zinc coordination is purely structural and may suggest that Zn^{2+} coordination is an essential element in promoting HIV-1 IN protein−protein interactions to generate the preintegration complex with the ends of the viral DNA (i.e., DNA looping formation), a phenomenon observed both with p53 and with the SV40 large T antigen.

ADDED IN PROOF

Engleman and Craigie (1995) have also recently reported that the Mg^{2+}-dependent activity required higher concentrations of IN, and that this activity is stimulated by PEG and DMSO.

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