Subterminal viral DNA nucleotides as specific recognition signals for human immunodeficiency virus type 1 and visna virus integrases under magnesium-dependent conditions

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Many reports describe the characteristics of susceptible viral DNA substrates to various retroviral integrases during in vitro reactions in which manganese serves as the divalent cation cofactor for site-specific nicking. However, manganese is known to alter the specificity of some endonucleases and magnesium may be the divalent cation used during retroviral integration in vivo. To address these concerns, we identified conditions under which the integrases of human immunodeficiency virus type 1 and visna virus were optimally active with magnesium (the first time such activity was shown for visna virus integrase) and used these conditions to test the susceptibility of a series of oligodeoxynucleotide substrates. The data show that two base pairs immediately internal to the conserved CA dinucleotide near the termini of retroviral DNA are selectively recognized by the two integrases and that the final six base pairs of viral DNA contain sufficient sequence information for specific recognition and cleavage by each enzyme. The results validate the importance of the subterminal viral DNA positions even in the presence of magnesium and identify viral DNA positions that functionally interact with integrase. The data obtained under magnesium-dependent conditions, which were obtained with substrates containing single and multiple base-pair substitutions and two different retroviral integrases, are consistent with those previously obtained with manganese. Thus, the large body of manganese-dependent data identifying terminal viral DNA positions that are important in substrate recognition by various integrases likely reflects interactions that are biologically relevant.

Introduction

Integration of a DNA copy of the retroviral genome into host-cell chromosomal DNA is an essential step in retrovirus replication and the pathogenesis of retrovirus-related diseases [see Brown (1997) and Asante-Appiah & Skalka (1997)] for recent reviews of integration]. This recombination event is catalysed by the retroviral integrase (IN), which is an attractive target for the development of specific antiretroviral therapy. The IN enzyme acts on two types of DNA substrates: viral DNA and host DNA. The double-stranded viral DNA, which is copied from the viral RNA genome by reverse transcriptase, has two long terminal repeat (LTR) sequences. Each LTR is comprised of U3, R and U5 regions; thus, the upstream end of viral DNA begins with the U3 region of one LTR and the downstream end terminates with the U5 region of the other LTR (Fig. 1A). To mediate integration, IN catalyses two distinct reactions at each end of the viral DNA. First, IN removes two nucleotides that follow highly conserved CA bases typically located at positions 4 and 3 from the 3’ end of each DNA strand to create recessed 3’-hydroxyl ends; this site-specific endonuclease activity is referred to as processing. Next, IN inserts the processed viral DNA ends into host DNA in a sequence-independent manner across a small fixed-length stagger of four to six base pairs; this activity is referred to as DNA joining or strand transfer. Both of these activities can be modelled in vitro by using purified IN proteins and oligodeoxynucleotides designed to mimic either viral DNA end (Fig. 1B). In these systems, IN appropriately removes two nucleotides following the conserved CA (Katzman et al., 1989)
and inserts the processed ends into various sites of other oligonucleotides that act as surrogates for host DNA (Katz et al., 1990; Craigie et al., 1990).

Many investigators have examined the characteristics of susceptible viral DNA substrates to illuminate the specific interactions between IN and the viral DNA termini. Although early studies with avian myeloblastosis virus IN used oligonucleotide-based assays in which magnesium served as the divalent metal cofactor [reviewed with citations in Katzman et al., 1989], integrases subsequently purified from a variety of retrovirus systems often are inactive with Mg$^{2+}$. Moreover, all integrases exhibit greater activity when manganese is present in standard assays. As a result, there is a large amount of published data describing the requirements for susceptible substrates of human immunodeficiency virus type 1 (HIV-1) and several other integrases using Mn$^{2+}$ as the divalent cation [reviewed with citations in Katzman & Katz (1999)]. Given that Mn$^{2+}$ may alter the specificity of other endonucleases (Hsu & Berg, 1978; Vermote & Halford, 1992) and that Mg$^{2+}$ may be the relevant divalent cation within cells (Miller et al., 1995; Bujacz et al., 1997; Asante-Appiah & Skalka, 1997a), the biological relevance of these results is unclear. To date, only one publication has described sequence requirements for viral DNA susceptibility to processing by HIV-1 IN under conditions in which Mg$^{2+}$ served as the cofactor (Esposito & Craigie, 1998). In particular, these investigators studied a series of oligonucleotide substrates that contained single base-pair substitutions in the HIV-1 U5 sequence. In the current report, we validate and extend those observations by identifying in vitro conditions under which another retroviral integrase exhibits Mg$^{2+}$-dependent activity that is comparable to its activity with Mn$^{2+}$. More importantly, we use these conditions for comparative studies involving two integrases and substrates that contain single or multiple base-pair substitutions, with a particular focus on the viral DNA U3 terminus, and compare the results with those obtained using Mn$^{2+}$. Together, the data provide a more complete understanding of the roles of subterminal viral DNA positions in recognition by IN.

**Methods**

- **Purified integrases.** Proteins were purified from a bacterial expression system as described previously (Katzman & Sudol, 1994, 1995). The concentrations of the purified HIV-1 and visna virus integrases were 357 ng/µl and 367 ng/µl, respectively, or about 10 pmol/µl for each enzyme, as measured by comparison to Coomassie blue-stained standards following SDS–PAGE and densitometry of dried gels.

- **Oligonucleotides.** All oligodeoxynucleotides used as assay substrates were gel-purified following synthesis and again after being 5'-end-labelled with [γ-32P]ATP by T4 polynucleotide kinase (Katzman & Sudol, 1994). Sequences are indicated in the appropriate figures.

- **In vitro integrase assays.** Double-stranded DNA substrates were prepared by annealing the labelled strand with 4-fold excess unlabelled complementary oligonucleotide (Katzman & Sudol, 1994). Standard 10 µl reaction mixtures contained 0.5 pmol of double-stranded DNA, 25 mM Tris–HCl (pH 8.0), 10 mM diithiothreitol, 1 µl of IN or protein storage buffer, and MnCl$_2$ or MgCl$_2$ as indicated for each experiment. Reaction mixtures were incubated for 90 min at 37 °C and then stopped by addition of 10 µl loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and heating at 95 °C for 5 min.
Aliquots were loaded onto 20% polyacrylamide (acrylamide to methylene-bisacrylamide ratio, 19:1)–7 M urea denaturing gels, followed by electrophoresis at 75 W until the bromophenol blue dye had migrated 21 cm. Wet gels were autoradiographed at ~70 °C.

### Quantification of results.
The radioactivity of bands in wet gels was quantified with a Betascope (Betagen). Specific viral DNA cleavage, or processing, was calculated as \( \frac{\text{(c.p.m. of 16-mers)} - \text{total c.p.m. in lane)}}{\text{(c.p.m. of > 18-mers)}} \), with background corrections from analogous parts of a negative control lane; > 18-mers refers to products longer than substrate length, which form only following specific cleavage (Katz et al., 1990; Craigie et al., 1990; Bushman & Craigie, 1991). Use of total c.p.m. in the denominator best reflects conversion of substrate to specific product, but yields lower calculated cleavages than would be suggested by merely comparing 16-mer products to the remaining 18-mer substrates.

### Results
#### Conditions under which HIV-1 and visna virus INs are active with magnesium

We previously showed that HIV-1 IN and visna virus IN are highly and comparably active on oligonucleotide substrates derived from the HIV-1 U5 terminus when Mn\(^{2+}\) serves as the cofactor (Katzman & Sudol, 1995). Thus, we used this substrate to screen for conditions that would permit Mg\(^{2+}\)-dependent activity by each of these enzymes. Mg\(^{2+}\)-dependent activity generally is absent or minimal with our preparations of HIV-1 IN and visna virus IN. Although others have reported that polyethylene glycol 8000 (PEG) or zinc can stimulate the Mg\(^{2+}\)-dependent activity of HIV-1 IN (Engelman & Craigie, 1995; Zheng et al., 1996; Lee & Han, 1996; Lee et al., 1997), we could not demonstrate that our enzyme preparations were stimulated by either of these reagents and found inhibition of activity under some conditions (data not shown). In contrast, dimethyl sulfoxide (DMSO), which also has been reported to enhance Mg\(^{2+}\)-dependent activity of HIV-1 IN (Engelman & Craigie, 1995; Lee & Han, 1996), dramatically stimulated the Mg\(^{2+}\)-dependent activity of our HIV-1 and visna virus IN preparations in a dose-dependent manner (Fig. 2). Maximal Mg\(^{2+}\)-dependent processing activity for these enzyme preparations was exhibited when reaction mixtures contained 5–10 mM Mg\(^{2+}\) and 30–40% (v/v) DMSO; under these conditions, at least 40% of the 18-mer substrate was converted to the 16-mer product (Fig. 2, lower panel, lanes 7, 8, 15 and 16). This level of activity is comparable to that achieved with Mn\(^{2+}\) (Fig. 2, lower panel, lanes 3 and 11). Minimal or no Mg\(^{2+}\)-dependent processing activity was evident in the absence of DMSO (Fig. 2, lower panel, lanes 4 and 12). Titration experiments showed that \( \geq 0.1 \) mM Mn\(^{2+}\) would need to be present in the final reaction mixture to detect any Mn\(^{2+}\)-dependent IN activity; such an amount is unlikely to be provided as a contaminant of the DMSO used in these experiments. Moreover, DMSO alone did not support processing activity by HIV-1 IN or visna virus IN (Fig. 2, lower panel, lanes 2 and 10). This result definitively excludes the possibility that stimulation of Mg\(^{2+}\)-dependent activity by DMSO was due to contaminating Mn\(^{2+}\). Addition of PEG or Zn\(^{2+}\) to reactions conducted with Mg\(^{2+}\) and DMSO provided no significant benefit and sometimes diminished activity (data not shown).

DMSO also stimulated the Mg\(^{2+}\)-dependent DNA joining activity of both enzymes to create products longer than substrate length (Fig. 2, upper panel). However, the patterns of strand-transfer products obtained with Mg\(^{2+}\) differed from the patterns obtained with the same enzyme and substrate DNA when Mn\(^{2+}\) served as the divalent cation. For example, note the location of the most prominent band created by HIV-1 IN in the presence of Mn\(^{2+}\) as compared with the most prominent band created with Mg\(^{2+}\) (Fig. 2, upper panel, the asterisks next to lanes 3 and 8, respectively). Similarly, visna virus IN created a predominant band with Mn\(^{2+}\) but two prominent bands with Mg\(^{2+}\) (Fig. 2, upper panel, the arrowheads next to lanes 11 and 16, respectively).

Exploration of one other approach to stimulating the Mg\(^{2+}\)-dependent activity of these enzymes provided additional important information. Although we confirmed the work of Lee et al. (1995a, b) that longer oligonucleotide substrates alter the divalent cation preference of HIV-1 IN, a similar result was not obtained for visna virus IN. In particular, double-stranded 18-mers from the HIV-1 U5 end were more susceptible to HIV-1 IN in the presence of Mn\(^{2+}\) than in the presence of Mg\(^{2+}\) (Fig. 3, lower panel, lanes 2 and 3), whereas 32-mers derived from the same viral DNA end were slightly more susceptible to HIV-1 IN with Mg\(^{2+}\) (Fig. 3, upper panel, lanes 2 and 3). In contrast, visna virus IN was inactive for processing on either substrate with Mg\(^{2+}\), although it preferred the shorter substrate with Mn\(^{2+}\) (Fig. 3, lanes 5 and 6). Thus, lengthening the size of the viral DNA substrate is not sufficient to reveal Mg\(^{2+}\)-dependent processing by all integrases. However, addition of DMSO dramatically stimulated the Mg\(^{2+}\)-dependent activity of visna virus IN on both substrates, especially the 18-mers (Fig. 3, lane 7). Although DMSO also stimulated the Mg\(^{2+}\)-dependent activity of HIV-1 IN on the shorter substrate, it did not do so on the longer one (Fig. 3, lane 4), perhaps because the level of activity on this substrate was high already. Because our preparations of HIV-1 IN and visna virus IN showed greatest Mg\(^{2+}\)-dependent activity when the substrates were 18 base pairs long and DMSO was present (Fig. 3, lower panel, lanes 4 and 7), we chose these conditions for subsequent experiments directed at analysing the role of subterminal nucleotides in recognition by IN. These conditions have the additional advantage of permitting direct comparisons with our previous results that were obtained using 18-mer substrates in the presence of Mn\(^{2+}\) (Katzman & Sudol, 1996).

Retroviral integrases generally exhibit different levels of activity on substrates derived from the two ends of unintegrated viral DNA, perhaps reflecting a need to process the two viral DNA ends sequentially rather than simultaneously (Kukolj & Skalka, 1995). In addition, some integrases are more
Fig. 2. Stimulation of Mg\(^{2+}\)-dependent activity by DMSO. Autoradiograms of denaturing polyacrylamide gels are shown. The lower panel is a short exposure of the lower part of one gel and the upper panel is a longer exposure of the upper part of the same reaction lanes but from a different gel. The regions overlap because both panels include the 18-mer substrate, as indicated at the left; the 16-mer processing product is indicated also. Double-stranded 18-mers derived from the U5 end of HIV-1 DNA were 5'-labelled on the strand that contains the conserved CA and incubated with protein buffer (lanes 1 and 9), HIV-1 IN (lanes 2–8) or visna virus IN (lanes 10–16). Reactions for lanes 1, 3, 9 and 11 contained 10 mM MnCl\(_2\) and no DMSO; reactions for lanes 2 and 10 contained no divalent cation but 30% DMSO; reactions for lanes 4–8 contained 7.5 mM MgCl\(_2\) and the indicated amounts of DMSO; and reactions for lanes 12–16 contained 10 mM MgCl\(_2\) and the indicated amounts of DMSO. The smears in the upper portions of lanes 1 and 9 are artefactual; no bands were evident in shorter exposures of these lanes. Prominent strand-transfer products created by HIV-1 IN or visna virus IN are highlighted with asterisks or arrowheads, respectively.

Active on heterologous substrates (Katzman & Sudol, 1995; Balakrishnan & Jonsson, 1997). Thus, we tested HIV-1 IN and visna virus IN under optimal Mg\(^{2+}\)-dependent conditions on all four possible viral DNA substrates, i.e. 18-mers derived from the U3 or U5 ends of HIV-1 or visna virus (as in Fig. 1A). The results were identical to those obtained when Mn\(^{2+}\) served as the divalent cation (Katzman & Sudol, 1995). In particular, both enzymes were very active on the HIV-1 U5 substrate (Fig. 4, lower panel, lanes 1–5) and less active on the visna virus U5 substrate (lanes 11–15). In addition, the two U3 substrates clearly differentiated between the two enzymes because each IN was much more active on its cognate U3 substrate (Fig. 4, lower panel, lanes 6–10 and 16–20). Moreover, each enzyme performed DNA joining on all four substrates (Fig. 4, upper panel) to the same extent as observed with Mn\(^{2+}\) (not shown), although the efficiency and strand-transfer patterns were dependent on the particular combination of enzyme and substrate, as observed previously (Katzman & Sudol, 1995). As noted above, the patterns of joined products, which reflect the preferential selection of target sites for insertion, also differed as a function of the divalent cation (data not shown). Because the enzymes exhibited markedly different processing efficiencies on the two U3 substrates, manipulation of these sequences offered a way to identify the viral DNA positions that are recognized by IN.

Positions 5 and 6 in U3 substrates act as recognition signals for both INs

Despite the different activities of each enzyme on the two U3 substrates, the U3 sequences differ at only two of their final nine base pairs, i.e. positions 5 and 6 (Figs 1A and 5C). Thus, we tested whether exchanging positions 5 and 6 in the U3 substrates would alter their susceptibility under optimal Mg\(^{2+}\)-
The above data demonstrate that positions 5 and 6 act as recognition signals for HIV-1 and visna virus integrases in the context of the U3 termini when Mg\(^{2+}\) serves as the cofactor, much as they were previously shown to function in the presence of Mn\(^{2+}\) (Katzman & Sudol, 1996). These data also suggest that only the last nine base pairs specifically interact with IN. However, positions 11 and 14 of the two wild-type U3 sequences are the same (Figs 1A and 5C) and may have contributed to the susceptibility of the various substrates to the enzymes. To exclude a sequence-specific role for the latter two positions, we mutated them in the context of the HIV U3s5,6 sequence (designated HIV U3s5,6,m11,14, where ‘m’ indicates a mutation rather than exchange substitution). This substrate was processed similarly to the visna U3 and the HIV U3s5,6 substrates when tested against the two integrases in the presence of Mg\(^{2+}\) (the last entry in Fig. 5C). Thus, positions 11 and 14 did not interact with these enzymes in a sequence-specific manner. We conclude from the data of Fig. 5, combined with the observation that the wild-type U3 substrates differ at positions 10, 12, 13 and 15–18, that all of the specific U3 information necessary for Mg\(^{2+}\)-dependent processing by either enzyme is contained in the last nine base pairs of the substrate. Furthermore, positions 5 and 6 are particularly important for recognition.

**Positions 5 and 6 in U3 substrates are recognized differently by the two INs**

To ascertain whether positions 5 and 6 played equal roles in Mg\(^{2+}\)-dependent recognition and cleavage by IN, these positions were independently exchanged between the HIV-1 and visna virus U3 substrates. The results of testing these substrates revealed that visna virus IN was more sensitive to changes in position 5, immediately adjacent to the invariant CA bases, whereas HIV-1 IN was more sensitive to changes in position 6. Thus, a substrate in which position 5 in the visna virus U3 substrate was substituted by the corresponding HIV-1 U3 base pair (designated visna U3s5) was processed significantly less efficiently by visna virus IN, and a substrate in which position 6 was substituted (designated visna U3s6) remained highly susceptible to this enzyme (Fig. 5B, lanes 14/15 and 19/20 compared with Fig. 5A, lanes 9/10). In contrast, the analogous HIV-1 U3s6 was not as hindered for susceptibility to HIV-1 IN as was the HIV-1 U3s6 substrate (Fig. 5B, lanes 2/3 and 7/8 compared with Fig. 5A, lanes 2/3; the differences are more evident when quantified and presented as in Fig. 5C, as discussed below). Note that the HIV-1 U3s5 and visna U3s6 substrates share the final nine positions and acted similarly in these assays (Fig. 5B, lanes 1–5 and 16–20). Similarly, HIV-1 U3s6 and visna U3s5 share their final nine positions and exhibited the same pattern of susceptibility to the two enzymes (Fig. 5B, lanes 6–10 and 11–15). The
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**Fig. 4.** Mg$^{2+}$-dependent activity of HIV-1 and visna virus INs on substrates from either DNA end of both viruses. The upper panel is a longer autoradiographic exposure of the upper part of a denaturing polyacrylamide gel and the lower panel is a shorter exposure of the lower part of the same gel (both panels include the 18-mer substrates). Each set of five lanes with the indicated substrates includes a negative control reaction incubated with protein buffer (−) and duplicate reactions with HIV-1 IN (H) or visna virus IN (V). Reactions with HIV-1 IN were conducted with 7.5 mM Mg$^{2+}$ and 30% DMSO and those for visna virus IN or the control buffer had 10 mM Mg$^{2+}$ and 35% DMSO. The positions of the 18-mer substrates and 16-mer products are indicated for the outer sets of reactions; note that the migration of the various oligonucleotides differs slightly as a function of sequence.

amounts (but not the patterns) of strand-transfer products were similar for substrates that matched at the final nine positions also (Fig. 5B, upper parts of lanes). The sequences of the singly substituted DNA substrates and quantification of multiple replicate reactions performed on different days are shown in Fig. 5C. This presentation highlights that the patterns of relative susceptibility to the two integrases are similar for HIV-1 U3s5 and visna U3s6 (the fifth and eighth entries in the figure), as well as for HIV-1 U3s6 and visna U3s5 (the sixth and seventh entries). Moreover, comparing results for a single enzyme on different substrates clearly demonstrates that visna virus IN was more dependent on position 5 than position 6 (compare its activity on visna U3, visna U3s5, and visna U3s6), whereas HIV-1 IN interacted more critically with position 6 (compare its activity on HIV-1 U3, HIV-1 U3s5, and HIV-1 U3s6). These results are identical to those obtained with Mn$^{2+}$ as the divalent cation (Katzman & Sudol, 1996).

**Substitutions in U5 substrates**

The above results reveal the importance of the final nine positions at the viral DNA U3 terminus for Mg$^{2+}$-dependent functional interactions with IN. To assess the importance of the final nine positions in the context of the U5 terminus when Mg$^{2+}$ serves as the cofactor, we tested an HIV-1 U5-derived oligonucleotide substrate in which positions 10–18 were mutated to base pairs found at none of the terminal DNA sequences from either virus (as shown in Fig. 1A). We found that this substrate (designated HIV-1 U5m10–18), as was the wild-type HIV-1 U5 sequence, was highly susceptible to both integrases (Fig. 6A). We then used a substrate in which every position but the final six was mutated (designated HIV-1 U5m7–18). This substrate also was susceptible to both integrases (Fig. 6B). Thus, as was observed with Mn$^{2+}$ (Katzman & Sudol, 1996), the final six base pairs contain sufficient information for specific processing by either IN. Nonetheless, the latter substrate was less susceptible than the wild-type sequence (compare Fig. 6B with Fig. 4, lanes 1–5, lower panel) and the susceptibility to visna virus IN was affected to a greater extent. Although these observations also were true for reactions conducted with Mn$^{2+}$ (Katzman & Sudol, 1996), the difference for visna virus IN was greater with Mg$^{2+}$. The sequences of the substrates and the results of multiple replicate reactions are summarized in Fig. 6(C).
Recognition of retroviral DNA by integrase

Discussion

Understanding how IN recognizes its DNA substrates is important for modelling retroviral integration and for suggesting new targets for antiretroviral therapy. Most published studies of specific recognition of the viral DNA termini by HIV-1 IN used Mn\(^{2+}\) as the divalent cation for the simple reason that many preparations of HIV-1 IN have little or no activity with Mg\(^{2+}\) when assayed under standard reaction conditions. However, Mg\(^{2+}\) is more abundant within cells and may be the relevant divalent cation in vivo. In addition, Mg\(^{2+}\) supports integration activity by preintegration complexes extracted from cells infected with HIV-1 (Farnet & Haseltine, 1990; Ellison et al., 1990) or murine leukaemia virus (Brown et al., 1987; Fujiwara & Mizuuchi, 1988). Moreover, Mn\(^{2+}\) can relax or alter the specificity of restriction endonucleases (Hsu & Berg, 1978; Vermote & Halford, 1992). These facts could raise questions about the biological relevance

![Fig. 5. Effects of U3 base substitutions. (A, B) Autoradiograms of denaturing polyacrylamide gels are shown for IN assays using the indicated substrates. Details are as in the legend to Fig. 4. (C) Quantification of results from multiple experiments. Sequences and numbering of substrates, as described in the text, are shown for the strand that contains the CA dinucleotide (shown in boldface). Upper-case letters indicate wild-type sequence or exchange substitutions, lower-case letters are used for other mutations, and dashes denote identity to the base in the HIV-1 U3 sequence. Specific cleavage measured in replicate experiments is shown as the mean ± standard error (error bar); each reaction was conducted an average of seven times.](image)
Fig. 6. Effects of U5 base substitutions. (A, B) Autoradiograms of denaturing polyacrylamide gels are shown for IN assays using the indicated substrates. Similar reactions for the wild-type HIV-1 U5 sequence (as in Fig. 4) are not shown. Details are as in the legend to Fig. 4. (C) Quantification of results from multiple experiments. Sequences of substrates are shown relative to the HIV-1 U5 sequence; details are as in the legend to Fig. 5(C). Specific cleavage measured in replicate experiments is shown as the mean ± standard error (error bar); each reaction was conducted an average of nine times.

of much of the published data that have defined functional interactions between IN and particular positions at the ends of viral DNA. This report should allay many of these concerns. Our new data obtained with Mg$^{2+}$ as the divalent cation confirm the key results previously demonstrated with Mn$^{2+}$, i.e. two base pairs just internal to the invariant CA bases near the ends of viral DNA act as specific recognition signals for two different lentiviral integrases.

Recently, several groups identified conditions under which HIV-1 IN has enhanced activity with Mg$^{2+}$. In addition to DMSO, factors reported to increase the Mg$^{2+}$-dependent activity of HIV-1 IN include: use of longer oligonucleotide substrates (Engelman & Craigie, 1995; Lee et al., 1995a, b), high enzyme-substrate ratios (Engelman & Craigie, 1995; Esposito & Craigie, 1998), or the presence of PEG (Engelman & Craigie, 1998), zinc (Zheng et al., 1996; Lee & Han, 1996; Lee et al., 1997), dioxane (Goodarzi et al., 1995), or the HIV-1 nucleocapsid protein (Carteau et al., 1997). Our results indicate that these conditions may not be uniformly effective for revealing activity by different integrases or enzyme preparations with Mg$^{2+}$. Although we confirmed that longer substrates enhance the Mg$^{2+}$-dependent activity of HIV-1 IN, a similar effect was not observed for visna virus IN (Fig. 3). We also were unable to stimulate the Mg$^{2+}$-dependent activity of our HIV-1 or visna virus enzyme preparations with PEG or Zn$^{2+}$ and found that these reagents inhibited activity in some reactions. Others have presented evidence that PEG can inhibit activity with Mn$^{2+}$ (Engelman & Craigie, 1995; Lee & Han, 1996) and that Zn$^{2+}$ inhibits the DNA joining activity of HIV-1 IN (Wolfe et al., 1996) and a nicking activity of avian sarcoma virus IN (Bujacz et al., 1997). In contrast, the organic solvent DMSO dramatically stimulated the Mg$^{2+}$-dependent processing and joining activity of both of our enzymes. In fact, 30–40% DMSO supported activity with Mg$^{2+}$ comparable to what is typically obtained with Mn$^{2+}$ (Fig. 2). These data represent the first demonstration that Mg$^{2+}$ can support activity by visna virus IN, a protein that has proven useful for constructing chimeric enzymes with HIV-1 IN (Katzman & Sudol, 1995, 1998). Others have noted that 10–20% DMSO can slightly enhance the Mg$^{2+}$-dependent processing activity of HIV-1 IN (Engelman & Craigie, 1995; Lee & Han, 1996) and feline immunodeficiency virus IN (Shibagaki et al., 1997). Interestingly, the HIV-1 IN preparation used by Esposito & Craigie (1998) readily exhibited Mg$^{2+}$-dependent processing with only 5% DMSO (along with 5% PEG). DMSO has also been reported to stimulate the Mg$^{2+}$-dependent integration activity of HIV-1 IN (Goodarzi et al., 1995; Miller et al., 1995), avian myeloblastosis virus IN (Fitzgerald et al., 1992; Vora & Grandgenett, 1995) and murine leukaemia virus IN (Fujiwara & Craigie, 1989).

How DMSO (or any of the other manoeuvres) promotes IN activity with Mg$^{2+}$ is unknown. DMSO can affect protein
secondary structure by two possible mechanisms (Jackson & Mantsch, 1991; Huang et al., 1995). When DMSO replaces solvent water molecules, the protein’s environment becomes less polar and hydrophobic interactions between nonpolar amino acid residues may be weakened. In addition, DMSO–IN interactions may replace hydrogen bonds at the protein surface or within interior cavities. Resulting effects on protein conformation may influence metal coordination by the enzyme’s active site residues (Maignan et al., 1998; Goldgur et al., 1998) or the stability of IN–substrate interactions in the presence of Mg\(^{2+}\) (Pemberton et al., 1996). Whatever the mechanism, it is significant that IN retains high specificity for the processing site near viral DNA ends in reactions conducted with DMSO and Mg\(^{2+}\) (Fig. 2). The true biological relevance of various reaction conditions is unclear because the environment in which IN operates within the cell is poorly defined. For example, the dilute aqueous reaction conditions typically used in vitro are unlikely to mimic intracellular conditions (Fulton, 1982; Zimmerman & Minton, 1993; Knurl & Minton, 1996). Although we do not claim that the presence of 30–40% DMSO is physiological, it is noteworthy that proteins may constitute a similar percentage of the weight of the cytoplasm in some cells (Fulton, 1982). Given the complexity of the intracellular milieu, the effects of molecular crowding on IN function deserve further study (Zimmerman & Minton, 1993; Minton, 1998). Nonetheless, the important point is that conditions were identified that provided a useful and productive means to investigate the role of subterminal viral DNA nucleotides as specific recognition signals for the HIV-1 and visna virus integrases in the presence of Mg\(^{2+}\). The data demonstrate that this particular parameter (i.e. the choice of divalent cation) does not affect recognition of certain key elements of the viral DNA substrate.

A major result from these experiments is that exchange of positions 5 and 6 between U3 substrates switched the patterns of susceptibility to two lenti viral integrases (Fig. 5). Positions 5 and 6 were also important for Mg\(^{2+}\)-dependent oligonucleotide processing by avian retroviral IN (Katzman et al., 1989; Cobrinik et al., 1991). Thus, interaction with these positions may be a general feature of retroviral integrases. However, integrases can differ with respect to the importance of these positions, as HIV-1 IN was more sensitive to substitution of U3 position 6 and visna virus IN was more dependent on position 5. These results complement those of Esposito & Craigie (1998), who found that the two base pairs just internal to the conserved CA bases play an important role in processing of U5 substrates by HIV-1 IN, that this effect was independent of the choice of divalent cation, and that position 6 had a greater influence on susceptibility to HIV-1 IN. Thus, IN likely interacts with positions 5 and 6 at both viral DNA termini in a similar fashion. Of note, Esposito & Craigie (1998) found that positions approximately one turn along the DNA helix from the conserved CA also affected susceptibility, but only when Mg\(^{2+}\) was the divalent cation. Their results from an in vitro selection assay and photocross-linking studies also suggested roles for these more-internal positions only when Mg\(^{2+}\) was present. Our data also suggest that positions 7–18 of viral DNA can influence the activity of IN, an effect that was greatest for visna virus IN with Mg\(^{2+}\) (Fig. 6) compared with Mn\(^{2+}\) (Katzman & Sudol, 1996). One difference between our results is that Esposito & Craigie found a large effect on Mg\(^{2+}\)-dependent processing when HIV-1 U5 position 12 or 13 (using our numbering) was substituted, whereas we found only minor effects when these positions, along with several others, were simultaneously replaced in the HIV U5m10–18 substrate (Fig. 6). This result suggests that other positions within this region can compensate for any effects on susceptibility to IN. It is not surprising that positions outside the terminal six base pairs can affect the extent of the reaction because at least 15 base pairs of substrate DNA are needed for optimal IN activity (Katzman et al., 1989; LaFemina et al., 1991; Sherman et al., 1992). Nonetheless, the final six U5 base pairs of viral DNA contain sufficient sequence information for specific recognition and cleavage in reactions conducted with Mg\(^{2+}\) (Fig. 6), Mn\(^{2+}\) (Katzman & Sudol, 1996) or both Mg\(^{2+}\) and Mn\(^{2+}\) (Sherman et al., 1992).

In contrast to the above findings, the choice of divalent cation appears to have a larger effect on interactions with the target for viral DNA insertion. Thus, the patterns of joined products for a given enzyme, donor DNA and target DNA sequence differed as a function of the metal (Fig. 2). The data of other groups also demonstrate that the strand-transfer patterns created by HIV-1 IN depend on whether Mg\(^{2+}\) or Mn\(^{2+}\) serves as the cofactor (Engelman & Craigie, 1995; Lee et al., 1995 b; Lee & Han, 1996); a similar result occurred with feline immunodeficiency virus IN (Shibagaki et al., 1997). Moreover, when we used a PCR-based assay to monitor insertion of processed viral DNA ends into a plasmid DNA target, the insertion patterns created by HIV-1 IN and visna virus IN differed for reactions conducted with Mn\(^{2+}\) compared with those conducted with Mg\(^{2+}\) (data not shown). That the nature of the divalent cation differentially affects interactions with viral DNA and host DNA suggests that IN has different binding sites for these substrates.

The most important finding in this study is that the role of subterminal viral DNA positions in recognition by the integrases of HIV-1 and visna virus was confirmed when Mg\(^{2+}\) substituted for Mn\(^{2+}\) during in vitro reactions. Thus, the choice of divalent cation is not critical for interactions between IN and positions close to the conserved CA bases at the viral DNA termini. The generalizability of our findings is strengthened by the use of substrates that contained single and multiple base substitutions, sequences derived from either end of unintegrated viral DNA, and two different retroviral systems. Thus, the large body of Mn\(^{2+}\)-dependent biochemical data identifying terminal viral DNA positions that are important in substrate recognition by various integrases likely describe biologically relevant interactions. The only caveat, as originally
suggested by Esposito & Craigie (1998), is that the role of more-internal viral DNA positions may have been underestimated in reactions conducted with Mn$^{2+}$. These functional data will permit correlations with structural information when the viral DNA binding site of IN is identified.

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References


Recognition of retroviral DNA by integrase


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