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The Role of Manganese in Promoting Multimerization and Assembly of Human Immunodeficiency Virus Type 1 Integrase as a Catalytically Active Complex on Immobilized Long Terminal Repeat Substrates

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The integration of a DNA copy of the viral genome into the genome of the host cell is an essential step in the replication of all retroviruses. Integration requires two discrete biochemical reactions: specific processing of each viral long terminal repeat terminus or donor substrate, and a DNA strand transfer step wherein the processed donor substrate is joined to a nonspecific target DNA. Both reactions are catalyzed by a virally encoded enzyme, integrase. A microtiter assay for the strand transfer activity of human immunodeficiency virus type 1 integrase which uses an immobilized oligonucleotide as the donor substrate was previously published (D. J. Hazuda, J. C. Hastings, A. L. Wolfe, and E. A. Emini, Nucleic Acids Res. 22:1121–1122, 1994). We now describe a series of modifications to the method which facilitate study of both the nature and the dynamics of the interaction between integrase and the donor DNA. The enzyme which binds to the immobilized donor is shown to be sufficient to catalyze strand transfer with target DNA substrates added subsequent to assembly; in the absence of the target substrate, the complex was retained on the donor in an enzymatically competent state. Assembly required high concentrations of divalent cation, with optimal activity achieved at 25 mM $MnCl_2$. In contrast, preassembled complexes catalyzed strand transfer equally efficiently in either 1 or 25 mM $MnCl_2$, indicating mechanistically distinct functions for the divalent cation in assembly and catalysis, respectively. Prior incubation of the enzyme in 25 mM $MnCl_2$ was shown to promote the multimerization of integrase in the absence of a DNA substrate and alleviate the requirement for high concentrations of divalent cation during assembly. The superphysiological requirement for $MnCl_2$ may, therefore, reflect an insufficiency for functional self-assembly in vitro. Subunits were observed to exchange during the assembly reaction, suggesting that multimerization can occur either before or coincident with but not after donor binding. These studies both validate and illustrate the utility of this novel methodology and suggest that the approach may be generally useful in characterizing other details of this biochemical reaction.

Integration is a step in the life cycle of retroviruses which is both uniquely characteristic and fundamental to retrovirus replication (reviewed in references 1, 20, and 38). Integration is necessary for stable maintenance of the retroviral genome and is also essential for high-level expression of viral genes (18, 29, 41). Two viral elements have been identified as absolutely required for integration: specific DNA sequences within the long terminal repeat (LTR) region at either end of the viral genome and the virally encoded enzyme integrase (7, 8–11, 22, 31–34).

Human immunodeficiency virus type 1 (HIV-1) integrase is packaged into virus particles along with two copies of the positive-sense RNA genome and reverse transcriptase. After reverse transcription, the double-stranded viral DNA is maintained as a highly ordered nucleoprotein complex within the cytoplasm of newly infected cells (2, 3, 14, 19, 28). The binding of integrase to specific sequences within each LTR and the processing of the viral termini constitute the first step in the overall integration reaction, 3′-end processing. This endonucleolytic cleavage reaction prepares the 3′ ends of the viral DNA for the subsequent strand transfer reaction by removing the terminal dinucleotide that is adjacent to bases within the LTR which are highly conserved and essential for catalysis. The next step in the reaction, strand transfer, occurs after the complex has migrated to the nucleus. Integrase generates a staggered nick on the cellular DNA and in a concerted reaction joins the 3′ ends of the processed viral termini to the 5′ ends of the nicked target DNA (reviewed in reference 1).

Integrase is the only viral enzyme known to be required to catalyze the steps of 3′-end processing and DNA strand transfer which define the integration reaction in vivo (10, 11, 32–34). During infection, these reactions are mediated by the enzyme as part of a preintegrative assembly containing additional viral and perhaps cellular components (2, 3, 14, 19, 28). The stability of this complex has been demonstrated in vivo as well as in vitro. In unactivated T-lymphoid cells, the preintegrative complex can persist in an integration-competent state for several weeks following HIV-1 infection (36, 42). In addition, preintegration complexes are isolated as stable assemblages from the cytoplasm of infected cells and retain their activity as nucleoprotein complexes throughout purification (3, 14). In vitro, preintegration complexes isolated from infected cells accurately recapitulate the integration reaction as is presumed to occur in vivo (14).

In vitro, 3′-end processing and the strand transfer of a single LTR end can be demonstrated, using recombinant enzyme and short oligonucleotides as substrates to represent both the donor and target DNAs in the reaction (5, 35). Recently, it has been shown that in vitro, as in vivo, the first step in the overall reaction pathway involves the assembly of a stable complex between integrase and the viral DNA donor substrate (15, 16, 40). In vitro, the formation of this complex requires either manganese or magnesium (15, 40).

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We previously described an assay system for HIV-1 integrase in which immobilized LTR oligonucleotides are used as the donor substrates for strand transfer (23). In this report, we describe studies in which these immobilized substrates are used to investigate the assembly and stability of the interaction between integrase and the donor DNA. We have demonstrated that this system can be used to dissect and reconstitute certain biochemical properties which are characteristic of complexes derived from HIV-1-infected cells, thereby validating the method both for the mechanistic study of integrase and for the discovery and analysis of relevant inhibitors of this potential antiviral target.

MATERIALS AND METHODS

Oligonucleotide DNA substrates. The oligonucleotides representing the HIV-1 HXB2 LTR sequences used to coat microtiter plates (Covalink; Nunc, Naperville, Ill.) and biotinylated oligonucleotides used as the target substrates were synthesized by Midland Certified Reagent Co. (Midland, Tex.).

Purification of recombinant wild-type and DQ mutant integrase. Cloning and expression of the wild-type and DQ mutant HIV-1 HXB2 integrases in *Escherichia coli* have been described elsewhere (29). Both proteins were purified by heparin-agarose chromatography and judged to be 85 to 90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously published (23). Protein concentrations were determined by dye binding as instructed by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

Microtiter assay using immobilized substrates. A microtiter assay for the strand transfer activity of HIV-1 integrase was published earlier (23). The assay was performed generally as outlined in Fig. 1, with any variations noted where appropriate.

The assay used an immobilized 30-bp U5 LTR sequence oligonucleotide (5′p ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CAGT 3′/3′ GAA AAT CAG TCA CAC CTT TTA GAG ATC GTC A 5′) as the virus-specific donor substrate in the reaction. When indicated, a preprocessed donor substrate (5′p ACC CTT TTA GTC AGT GTG GAA AAT CTC TAG CA 3′/3′ GAA AAT CAG TCA CAC CTT TTA GAG ATC GTC A 5′) was used. Microtiter plates were coated with either the unprocessed or preprocessed U5 oligonucleotide such that the amount of donor immobilized per well was 1.25 pmol (a final concentration of 12.5 nM in a 100-μl reaction mixture). For all experiments, a heterologous biotinylated 20-bp oligonucleotide (5′ TGA CCA AGG GCT AAT TCA CT 3′-biotin/biotin-3′ ACT GGT TCC CGA TTA AGT GA 5′) was used as the target substrate for strand transfer.

The assay was performed directly in the donor substrate-coated microtiter plate. Each 100-μl reaction mixture included 20 mM Tris-HCl (pH 7.8), 25 mM NaCl, 3 mM $MnCl_2$, 5 mM β-mercaptoethanol, and 50 μg of bovine serum albumin per ml. Binding and 3′-end processing were initiated by the addition of integrase (a final concentration of 50 nM was standard unless otherwise indicated). The times for binding and/or end processing were varied as outlined in relevant figure legends. When indicated, a removal/wash step was introduced between the binding step and the strand transfer reaction: following the removal of integrase, the wells were washed three times with 100 μl of the incubation buffer as appropriate for the experiment. For those experiments in which the conditions for the strand transfer reaction were distinct from binding conditions, a final rinse step in the corresponding strand transfer reaction buffer was also performed prior to initiation of the strand transfer reaction.

Reaction buffer (95 μl) was added back to each well, and strand transfer proceeded upon addition of the biotinylated target DNA substrate (5 μl at 3 μM, a final concentration of 150 nM). After incubation at 37°C, the assay was terminated by removing the unreacted target substrate. After extensive washing, the plates were blocked and strand transfer products were detected by using an avidin-linked alkaline phosphatase colorimetric reporter system as described previously (23). Optical densities were determined at 405 nm, using a microtiter plate reader.

Glutaraldehyde cross-linking and Western immunoblot analysis. Glutaraldehyde cross-linking studies were performed essentially as described previously (25), using concentrations of integrase and $MnCl_2$ as outlined in the relevant figure legends. The conditions for Western analysis using an antipeptide antibody directed against the C-terminal domain of integrase (amino acids 270 to 288) were as detailed in a previous publication (24). Reactions were analyzed by SDS-PAGE, using 10% Tricine gels and conditions recommended by the manufacturer (Novex, San Diego, Calif.).

RESULTS

The strand transfer complex assembles on the immobilized LTR donor substrate. The configuration of the microtiter strand transfer assay published previously (23) was modified for these studies as outlined in Fig. 1. This colorimetric assay uses an immobilized LTR oligonucleotide as the donor substrate and a biotinylated oligonucleotide as the target DNA substrate for strand transfer. Covalent incorporation of the biotinylated oligonucleotide is detected with avidin-alkaline phosphatase, and optical density is monitored as a measure of the extent of strand transfer. In the microtiter assay, the strand transfer reaction is sensitive to mutations in the donor substrate previously shown to impair function (23). In addition, the assay is strictly dependent on the concentrations of donor and target DNA substrates in the reaction and displays hyperbolic saturation with respect to the concentrations of both substrates (23).

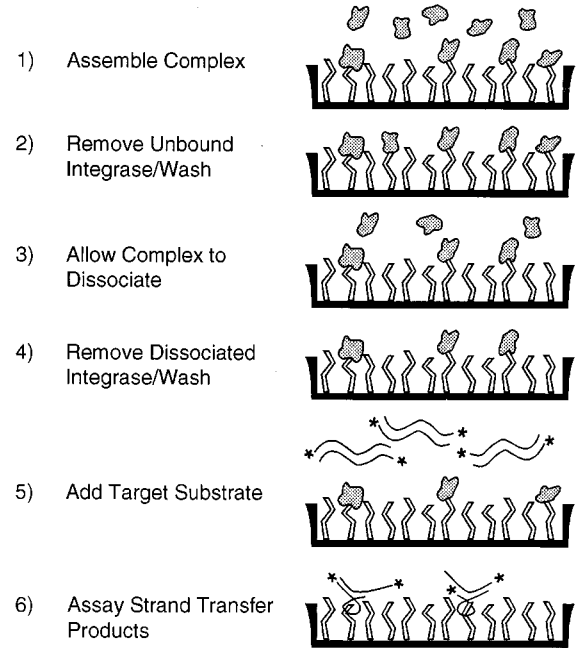

FIG. 1. Outline of the strand transfer assay using immobilized LTR oligonucleotides. The microtiter assay was performed as described previously (23), with the following modifications: the assembly time and/or buffer conditions were varied (step 1), and a wash step (step 2) was introduced and varied as appropriate. For stability experiments, an interim incubation was included subsequent to the assembly reaction (steps 3 and 4).

Since the assay uses distinct donor and target DNAs as substrates in the reaction, the processes of donor binding and 3′ endonucleolytic cleavage may be uncoupled from strand transfer. In addition, the use of an immobilized LTR donor provides a potential means of isolating and characterizing individual steps in the overall reaction. As diagrammed in Fig. 1, immobilization of the donor substrate permits removal of uncomplexed enzyme at any of several points in the reaction and thus may facilitate the study of donor binding and the stability of the integrase-donor substrate interaction, as well as strand transfer.

The first step in the reaction involves the binding of integrase to the immobilized LTR donor. To study the interaction between integrase and the LTR donor substrate independent of strand transfer, integrase was preincubated with the donor substrate for 30 min before initiation of the strand transfer reaction. In a subset of these reactions, any enzyme not bound during the preincubation period was removed and the complexes were washed extensively prior to the addition of the biotinylated target DNA substrate. Strand transfer was initiated by the addition of the target substrate, and the reaction

TABLE 1. Optical densities of donor substrates[a]

Donor substrate	Optical density (%)
Unprocessed	
Unwashed	1.164
Washed	1.064 (91)
Preprocessed	
Unwashed	1.020
Washed	0.950 (93)

[a] Microtiter plates were coated with either unprocessed or preprocessed LTR donor substrates, and the strand transfer assay was performed as outlined in Materials and Methods. After the 30-min incubation with the donor and before addition of the biotinylated target substrate as indicated, subsets of the samples were washed (three times, each in 100 μl of reaction buffer) to remove unassociated integrase. Each reaction was performed in triplicate, and the data represent averages of the optical density values at 405 nm.

mixtures were incubated for an additional 30 min. The amount of product in each case was quantified by assaying for the presence of the biotin tag.

As shown in Table 1, a comparison of the results from the washed and unwashed assays demonstrated that the reactions in which the target DNA substrate was added after any unbound enzyme had been removed were indistinguishable from the reactions wherein unassociated enzyme was potentially present. Therefore, excluding integrase bound to the donor substrate, additional enzyme was not required to mediate catalysis with the target DNA substrate. The enzyme which associated with the immobilized donor substrate was itself sufficient to catalyze the entire reaction, 3′-end processing as well as strand transfer.

Assembly of the strand transfer complex can be analyzed as a discrete step independent of catalysis. The data summarized in Table 1 demonstrate that in the absence of a target DNA substrate, integrase was stably and functionally maintained in association with the immobilized LTR. This was true for both unprocessed and preprocessed donors, i.e., oligonucleotides lacking the 3′-terminal dinucleotide which is normally removed by integrase. The assay conditions can therefore be manipulated without perturbing the integrase-donor substrate interaction, thus permitting the discrete analysis of the assembly and strand transfer processes. The assembly and stability of the integrase donor substrate complex and strand transfer were assayed under different conditions. In addition, we also monitored the kinetics of each of these reactions. For all experiments, the amount of enzyme productively associated with the donor was assessed by measuring strand transfer activity.

To study the kinetics of assembly, the preincubation time was varied from 5 to 60 min, any enzyme not bound to the donor substrate was then removed, and the strand transfer reaction was performed for 30 min as described above. The kinetics of assembly on both preprocessed and unprocessed donor substrates were evaluated, and the results are shown in Fig. 2A and B, respectively. Although the overall activities were equivalent for both the processed and unprocessed substrates, a slight but reproducible delay was observed when the unprocessed donor was used.

For comparison, the kinetics of strand transfer were also investigated. Complexes were assembled on preprocessed donors, excess integrase was removed, and the strand transfer reaction was initiated with target substrate. Strand transfer was allowed to proceed at 37°C, with the reaction time varied. The assays were terminated by removing the unreacted target substrate at each time and quantifying the biotinylated products. As shown in Fig. 2C, the kinetics of strand transfer approximated the kinetics previously observed for assembly on the unprocessed substrate (Fig. 2B), and both reactions were somewhat slower than observed for the kinetics of assembly on the preprocessed donor (Fig. 2A).

The reactions shown in Fig. 2B and C reflect events which encompass catalysis as well as substrate binding, and in each case the time to achieve half-maximal activity was approximately 12 min. However, when preprocessed donors were used, the kinetics represent the assembly reaction exclusively and the time to achieve half-maximal activity was less than 5 min (Fig. 2A). These data suggest that formation of the enzyme substrate complex may not be coincident with catalysis but that the binding of either the donor or target substrates may in fact precede both the 3′-end processing and strand transfer reactions. A comparative analysis of the kinetics of 3′-end processing and donor substrate binding in solution has also suggested that assembly and catalysis are distinct and processive steps in the reaction (15).

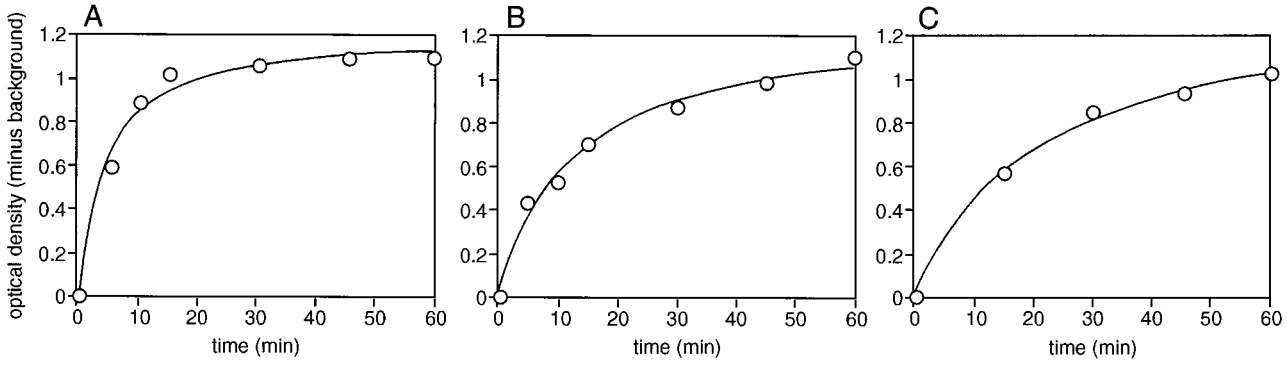

FIG. 2. Kinetics of assembly, end processing, and strand transfer. The reaction time for assembly (step 1) was varied as shown, using preprocessed and unprocessed donor substrates (A and B, respectively). Therefore, in panel A, the kinetics reflect solely the assembly reaction, whereas in panel B, the reaction is dependent on both assembly and 3′-end processing. At the times indicated, unassociated enzyme was removed as described previously, and strand transfer was initiated by the addition of the biotinylated target DNA substrate. The strand transfer reaction was performed for 30 min at 37°C. In panel C, the kinetics of strand transfer was monitored as follows. Complexes were assembled at 37°C for 30 min on preprocessed donors, unassociated enzyme was removed, and the target DNA was added. The reaction mixture was incubated at 37°C for the times indicated. The reaction was quenched by removing any unincorporated target substrate prior to analysis. In all panels, the amount of strand transfer was quantified by assaying for the incorporation of the biotin tag, using an avidin-linked alkaline phosphatase reporter, and measuring the optical density at 405 nm.

Divalent cation is required for both assembly and catalysis of the donor bound complex. Divalent cations have previously been shown to be essential for the 3′-end processing and strand transfer activities of integrase (35, 39). Although divalent cations are not essential for binding DNA nonspecifically (24, 30), it has recently been shown that either manganese or magnesium is required for productive binding and assembly of a stable complex between integrase and the donor DNA substrate (15, 40). This requirement for divalent cation has been observed not only with respect to the productive interaction of integrase with the donor DNA substrates in strand transfer but also for branched DNA substrates in disintegration (17a).

Therefore, for comparison, we investigated the requirement for manganese in assembly and strand transfer in the microtiter assay. These experiments were similar to those shown in Fig. 2. In addition, by introducing a variable interim incubation step between the assembly and strand transfer reactions, we also measured the half-life of the functional integrase-donor substrate association. For stability studies, we incubated preassembled complexes at 37°C for various times in the presence or absence of $MnCl_2$ before removing any potentially dissociated enzyme, replacing the $MnCl_2$, and initiating the reaction with the addition of the target DNA.

As shown in Fig. 3A, when $MnCl_2$ was omitted from either the initial binding step or the subsequent strand transfer reaction, biotinylated reaction products were not detected. This was true for unprocessed donor substrates as well as for preprocessed donors, suggesting that in the latter case, $MnCl_2$ was essential for assembling a functional strand transfer complex on the immobilized LTR. Although $MnCl_2$ was required for assembly, the continued presence of the divalent cation in solution was not required to maintain the stable association. Regardless of whether divalent cation was included in the interim incubation, the residual activities of the preassembled complex were comparable at all times as shown for up to 2 h (Fig. 3B). Therefore, manganese was not required in the incubation buffer to maintain the complex in a catalytically active state; however, as shown in Fig. 3, washing the complex in EDTA effectively abolished activity.

At 37°C, the half-life of the functional interaction was approximately 2.5 h (Fig. 3B). At 22 or 4°C, no loss of activity could be detected for greater than 4 h (data not shown). Complexes assembled on preprocessed donor substrates and those assembled on unprocessed donor substrates exhibited identical half-lives under all conditions (Fig. 3B) and were equally insensitive to disruption by incubation in concentrations of NaCl which prevent assembly (0.5 to 1 M; unpublished data). These observations suggest that processing affects neither the stability nor the avidity of the integrase-donor substrate interaction.

The requirement for divalent cation in assembly is functionally distinct from the requirement in catalysis. In Fig. 2 and 3, both assembly and catalysis were performed in 25 mM $MnCl_2$. Previous studies have demonstrated that the enzymatic functions of 3′-end processing and strand transfer require high concentrations of divalent cation for optimal activity (39). However, the $MnCl_2$ concentration dependence for either assembly or catalysis of strand transfer independent of donor substrate binding has not been addressed. We determined the $MnCl_2$ requirement for assembly by varying the concentration of $MnCl_2$ included during the binding reaction and then performing the strand transfer reaction in 25 mM $MnCl_2$ (Fig. 4A). The $MnCl_2$ requirement for catalysis was also determined by first assembling the integrase-donor complex in 25 mM $MnCl_2$ and, after extensive washing, varying the concentration of $MnCl_2$ in the strand transfer reaction (Fig. 4B).

The optimal concentration for $MnCl_2$ in assembly was approximately 25 mM, with little or no strand transfer activity detectable when the assembly reaction was performed in 1 mM $MnCl_2$ (Fig. 4A). However, when complexes were preassembled in 25 mM $MnCl_2$, the strand transfer reaction was equally efficient in either 1 or 25 mM $MnCl_2$ (Fig. 4B). Similar results were obtained for $MgCl_2$, although overall the reaction was significantly less efficient (data not shown).

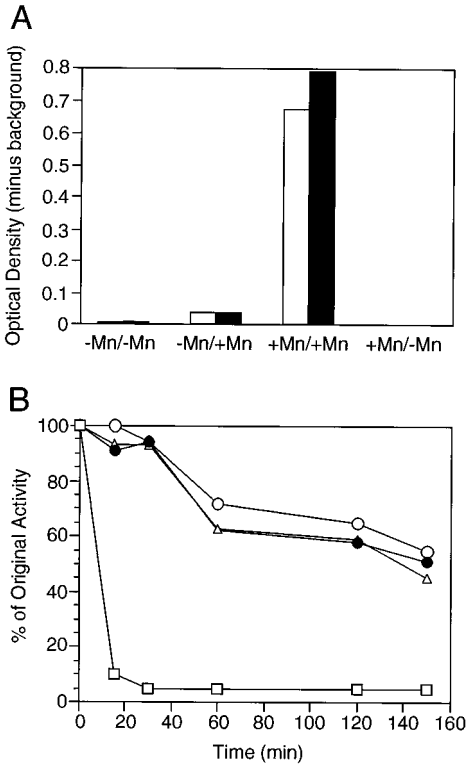

FIG. 3. Requirement for divalent cation during assembly and strand transfer. In panel A, the strand transfer assay was performed under standard conditions except that $MnCl_2$ was omitted during step 1 (binding and 3′-end processing) and/or step 5 (strand transfer). Both unprocessed and preprocessed donor substrates were used (open and solid bars, respectively). In panel B, stability of the integrase donor substrate complex was monitored. $MnCl_2$ at 25 mM was included during both the assembly and strand transfer reactions (steps 1 and 5); however, an additional interim incubation as shown in Fig. 1 (steps 3 and 4) was performed for the times indicated in either the presence (preprocessed and unprocessed donors; open triangles and circles, respectively) or absence (preprocessed donors only; solid circles) of 25 mM $MnCl_2$ or in the absence of $MnCl_2$ and in the presence of 25 mM EDTA (squares). Before and after the interim incubation, complexes were washed three times in $MnCl_2$-deficient reaction buffer before replacement of the $MnCl_2$ and initiation of strand transfer by the addition of the target substrate. Strand transfer activity is presented relative to the optical density of reactions in which steps 3 and 4 were omitted.

Preincubation of integrase with high concentrations of manganese alleviates the requirement for high concentrations of manganese during assembly. Since both the assembly and strand transfer reactions in Fig. 4 required de novo association with substrate DNA, the unique divalent cation optimum observed for each reaction suggests that the role of $MnCl_2$ during assembly may not directly involve interaction with the donor substrate. To examine the possibility that high concentrations of $MnCl_2$ were altering the activity of the enzyme itself, integrase was preincubated in the absence of donor substrate for 30 min at 37°C with either 2.5 or 25 mM $MnCl_2$, conditions previously shown to be inactive or optimal, respectively, in promoting assembly. To ensure that the contribution from the $MnCl_2$ in the preincubation would not significantly affect the

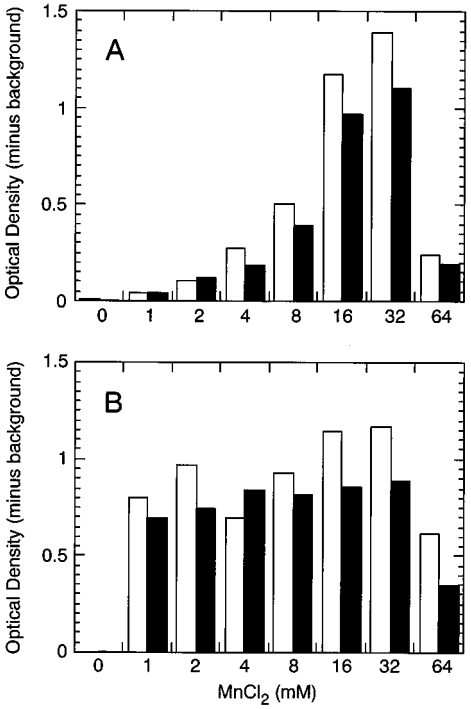

FIG. 4. Requirement for manganese in assembly and strand transfer. In panel A, the concentration dependence for $MnCl_2$ during assembly was investigated. Integrase was incubated with preprocessed donor substrates (open bars) or unprocessed donor substrates (solid bars) at 37°C for 30 min, with the concentration of $MnCl_2$ varied from 0 to 64 mM. Complexes were extensively washed, and the strand transfer reaction was performed under standard conditions in the presence of 25 mM $MnCl_2$. In panel B, the concentration dependence for $MnCl_2$ in strand transfer was studied by assembling complexes on either preprocessed or unprocessed donors (open or solid bars, respectively) at 37°C for 30 min in 25 mM $MnCl_2$. As in panel A, complexes were then washed and the strand transfer reaction was performed with the indicated concentrations of $MnCl_2$. In both panels, strand transfer products were quantified by assaying for the incorporation of the biotin tag, using an avidin-linked alkaline phosphatase reporter and measuring the optical density at 405 nm.

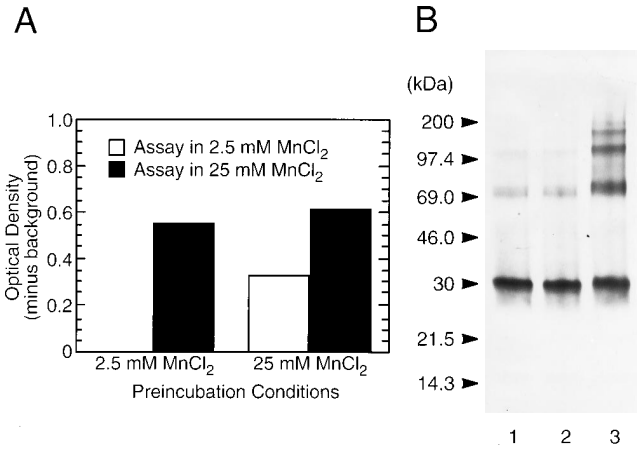

FIG. 5. Effect of manganese on the activity and multimerization of integrase. Integrase (500 nM) was preincubated at 37°C for 30 min in reaction buffer containing 100 mM NaCl with $MnCl_2$ as indicated. In panel A, the strand transfer activity of enzyme incubated in either 2.5 or 25 mM $MnCl_2$ was evaluated following dilution of the enzyme 10-fold into the reaction. Both the assembly and strand transfer reactions were performed in 2.5 or 25 mM $MnCl_2$ as indicated. Strand transfer activity was quantified as described for Fig. 4. In panel B, the multimeric form of the enzyme incubated in either 0, 2.5, or 25 mM $MnCl_2$ was analyzed by Western blotting. Integrase was preincubated as for panel A in the presence of 0, 2.5, or 25 mM $MnCl_2$ (lanes 1, 2, and 3, respectively). Each 50-μl reaction mixture was then precipitated by the addition of 100% trichloroacetic acid to a final concentration of 10% prior to separation by SDS-PAGE on 10% Tricine gels. Western blot analysis was performed with an antipeptide antibody to integrase (amino acids 270 to 288) as described previously (24).

subsequent reaction, the concentration of integrase used in the preincubation was 10-fold higher than the concentration used in the actual strand transfer assay. The preincubated enzyme could then be diluted 10-fold into the reaction, and the assay could be performed in a final concentration of either 2.5 or 25 mM $MnCl_2$.

Consistent with the results from the previous experiment, when integrase was preincubated in 2.5 mM $MnCl_2$, strand transfer activity was observed only when the reaction was performed in 25 mM $MnCl_2$, not when the assay was performed at 2.5 mM $MnCl_2$ (Fig. 5A). In contrast, preincubating the enzyme in 25 $MnCl_2$, the concentration optimal for assembly, produced a substantial increase in the strand transfer activity of integrase under conditions in which the enzyme was previously inactive. As shown in Fig. 5A, at 2.5 mM $MnCl_2$, the strand transfer activity of the enzyme preincubated in 25 mM $MnCl_2$ was recovered to greater than 50% of the activity observed when the assay was performed in 25 mM $MnCl_2$.

High manganese concentrations promote an increase in the multimerization state of integrase. The high divalent cation requirement for assembly of the integrase-donor substrate complex was considerably circumvented by preincubating the enzyme alone in high concentrations of $MnCl_2$, suggesting that $MnCl_2$ may have affected a structural change in the enzyme itself. Integrase forms multimers in solution (13, 16, 21, 27, 39) and can be cross-linked to DNA as either a monomer or multimer (12, 24, 30). It is also well established that the enzymatic functions of integrase, 3′-end processing as well as strand transfer, are accomplished by a multimeric form of the protein (16, 17, 26, 37). We therefore examined whether $MnCl_2$ alters the multimerization state of integrase in solution.

To examine the dependence of integrase multimerization on the presence of divalent cation, integrase was incubated as described above in either 0 or 25 mM $MnCl_2$. Samples were cross-linked with glutaraldehyde, separated by SDS-PAGE, and analyzed by densitometry (Fig. 6). As shown in Fig. 6A and C, integrase incubated in the absence of $MnCl_2$ was almost exclusively monomeric. This was true for both un-cross-linked and cross-linked samples, although the glutaraldehyde-treated protein migrated at an anomalously low molecular weight because of extensive intramolecular cross-linking. In contrast, incubation in the presence of 25 mM $MnCl_2$ induced a substantial increase in the relative amounts of several lower-mobility forms of the enzyme, and the emergence of these apparent multimeric products was correlated with a coordinate decrease in the monomer peak (Fig. 6B and D). In both the cross-linked and un-cross-linked samples, a significant peak of density appeared at a mobility which would be consistent with dimers of the enzyme (Fig. 6B and D). In addition, trace amounts of potential trimers and tetramers were visible in the glutaraldehyde-treated sample (Fig. 6D).

To demonstrate that the lower-mobility products observed in Fig. 6 were in fact multimers of integrase, samples were subjected to further analysis by Western blotting. When integrase was incubated in either the absence of $MnCl_2$ or the presence of 2.5 mM $MnCl_2$, the immunoreactive material migrated predominantly as a single band of approximately 32 kDa on SDS-PAGE (Fig. 5B, lanes 1 and 2). However, as was observed in Fig. 6B and D, incubation of integrase in 25 mM $MnCl_2$ induced the appearance of three new immunoreactive

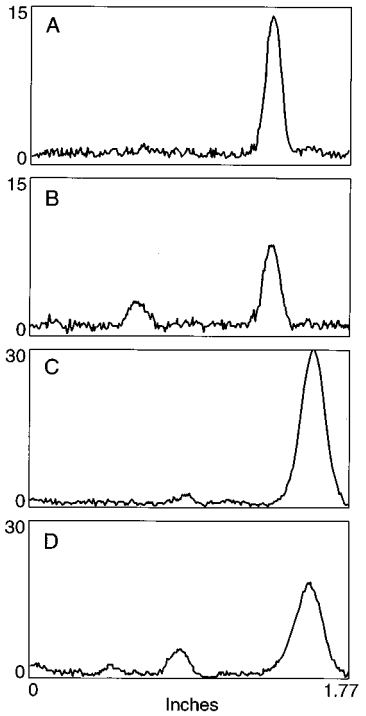

FIG. 6. Manganese increases the multimerization state of integrase. The effect of manganese on the multimerization state of integrase in solution was investigated by glutaraldehyde cross-linking. Reaction mixtures of 200 μl contained 1 μM integrase in the standard strand transfer reaction buffer containing 50 mM 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) and either no $MnCl_2$ (A and C) or 25 mM $MnCl_2$ (B and D). Samples were incubated at 37°C for 30 min and then cross-linked with glutaraldehyde for 5 min at 22°C essentially as described previously (25). After the reaction was quenched, samples were concentrated by trichloroacetic acid precipitation prior to analysis by SDS-PAGE on 10% Tricine gels. Gels were stained with Coomassie blue, and the bands were quantified by densitometric scanning. (A and B) Densitometric traces of un-cross-linked integrase incubated in 0 and 25 mM $MnCl_2$, respectively; (C and D) scans of the glutaraldehyde-treated enzyme incubated in 0 and 25 mM $MnCl_2$. One inch = 2.54 cm.

species whose apparent molecular masses of 68, 98, and 130 kDa were consistent with dimers, trimers, and tetramers of the enzyme (Fig. 5B, lane 3).

The donor-bound complex assembles as a multimer. The results in Fig. 4 to 6 demonstrated a correlation between the requirement for $MnCl_2$ in assembly and the ability to induce an increase in the overall multimerization state of integrase in solution. To further study the role of multimerization in assembly, a catalytically inactive enzyme, VE(151,152)DQ, expressing amino acid substitutions at residues 151 (V to E) and 152 (D to Q) was used in an attempt to incorporate both wild-type and mutant subunits into a multimeric complex having an enzymatically distinct and therefore biochemically trackable phenotype. These mutations occur in the highly conserved, active site of the enzyme, and this mutant enzyme has previously been shown to have less than 1% of the activity of the wild-type enzyme in both the 3′-end processing and strand transfer reactions (29). The mutant enzyme had no detectable activity in the microtiter assay (data not shown).

As shown in Fig. 7A, when the mutant integrase was mixed in increasing proportions with the wild-type enzyme immediately prior to binding of the donor substrate, strand transfer activity of the resultant complex was reduced in a dose-dependent manner. These results suggest that a complex composed of a mixed-subunit multimer with diminished enzymatic activity was formed immediately prior to or concomitant with donor substrate binding. A comparable decrease in strand transfer activity was elicited for each combination when the enzyme mixture was preincubated for 30 min prior to assembly (data not shown).

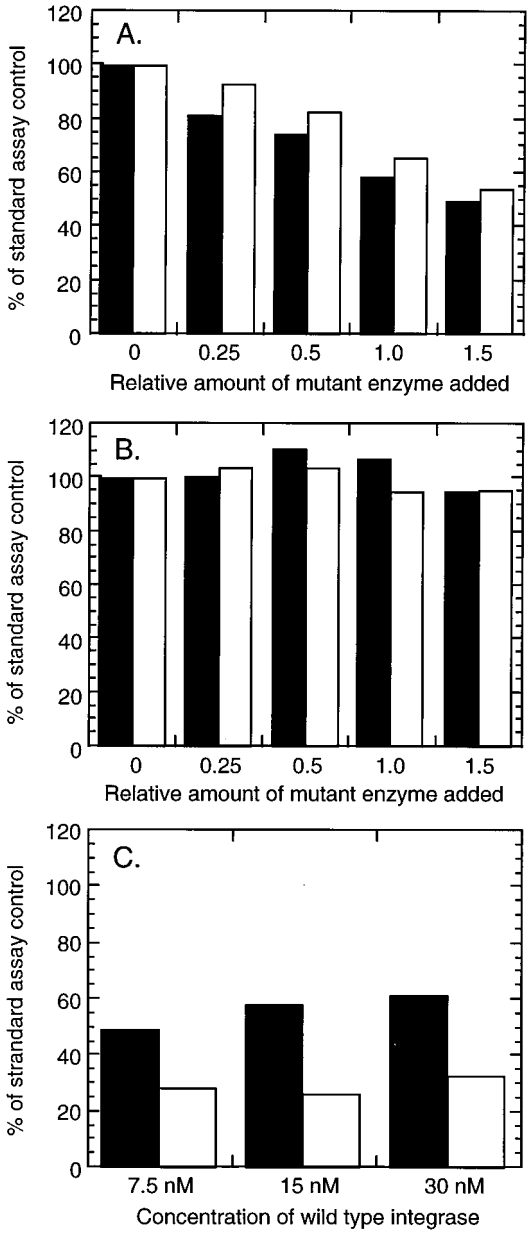

FIG. 7. Mixed multimers are formed during but not after assembly. Assembly reactions were performed at 37°C, using the standard reaction buffer with both preprocessed (open bars) and unprocessed (solid bars) donor substrates. In panel A, complexes were assembled for 30 min, using 25 nM integrase in the presence of increasing concentrations of the mutant enzyme (see text). In panel B, reactions were preassembled by using 25 nM wild-type enzyme and then washed extensively before addition of the mutant enzyme in the same proportions as in panel A. The reaction mixtures were then incubated for an additional 30 min at 37°C before removal of the reaction mix and performance of strand transfer. In panel C, proteins were mixed at a ratio of either 1:1 or 1:2 (wild-type to mutant enzyme; solid or open bars, respectively) immediately prior to binding of the donor substrate. The mixes were diluted into the reaction mixture such that the concentration of wild-type integrase was 7.5, 15, or 30 nM as indicated.

Although the experiment shown in Fig. 7A was performed

with suboptimal concentrations of integrase (see Fig. 8), it remained possible that the dose-dependent decrease in activity could be trivially due to competition by the mutant enzyme for available substrate. Therefore, mutant enzyme was premixed with the wild-type integrase at two different ratios which significantly reduce strand transfer (1:1 and 1:2, wild type to mutant). These mixes were then titrated in the microtiter assay such that the final concentration of the wild-type enzyme varied from 7.5 to 30 nM. As shown in Fig. 7C, inhibition was dependent only on the relative amount of the mutant enzyme added in the reaction and was independent of total enzyme concentration, indicating that the mutant protein does not reduce activity by titrating out the available substrate but does so by titrating out the enzymatically competent integrase.

In contrast to the results in Fig. 7A and C, when the VE(151,152)DQ mutant was added in the same proportion to wild-type complexes which had been preassembled on the donor substrate, no loss in activity was observed, even with the highest concentration of mutant enzyme (Fig. 7B). The strand transfer complex is therefore composed of a stable multimer in which the subunits associate in solution prior to or upon binding to the LTR but are not readily exchanged subsequent to assembly. These data also suggest that a functional strand transfer reaction may require the participation of more than one enzymatically competent active site.

Assembly of the strand transfer complex is time and protein concentration dependent. The observation that the strand transfer complex assembles as a multimer suggests that assembly on the immobilized donor substrate should be time and protein concentration dependent. Protein concentration dependence was examined as a function of time by incubating different concentrations of integrase with the preprocessed donor substrate for various times from 5 min to 2 h. The amount of integrase productively associated with the immobilized LTR at each time was then assessed as in previous experiments by removing unassociated enzyme and assaying the strand transfer activity of the resultant complex.

At each concentration of integrase, assembly of the complex was fairly rapid, with maximal activity for the standard enzyme concentration (50 nM) attained between 15 and 30 min as was shown in Fig. 2 (Fig. 8). At a higher concentration of integrase, the assembly reaction plateaued sooner, whereas at lower enzyme concentrations, an increase in strand transfer activity was achieved with prolonged incubation. At all concentrations of integrase, however, no significant increase in strand transfer activity was detected when assembly reaction mixtures were incubated for greater than 60 min.

As shown in Fig. 8, strand transfer activity was observed to reach maximal levels at a concentration of integrase equivalent to 50 nM monomer (Fig. 8). Although increasing the concentration of integrase to 100 nM accelerated that kinetics of assembly, the maximum amount of activity produced in these reactions was equivalent to that in assays assembled with 50 nM enzyme. The format of the microtiter strand transfer assay precludes enzyme turnover, and the level of strand transfer activity is constrained by the amount of immobilized donor substrate. Saturation may therefore reflect the quantity of enzyme which can be accommodated by the concentration of donor substrate in the reaction. If the enzyme is fully competent and given that the concentration of immobilized donor substrate was 12.5 nM, a concentration of 50 nM would be consistent with integrase assembling as a tetramer. However, we cannot exclude the possibility that this concentration of enzyme is required drive multimerization and may therefore simply reflect the affinity of the enzyme for self-association in the presence of donor substrate.

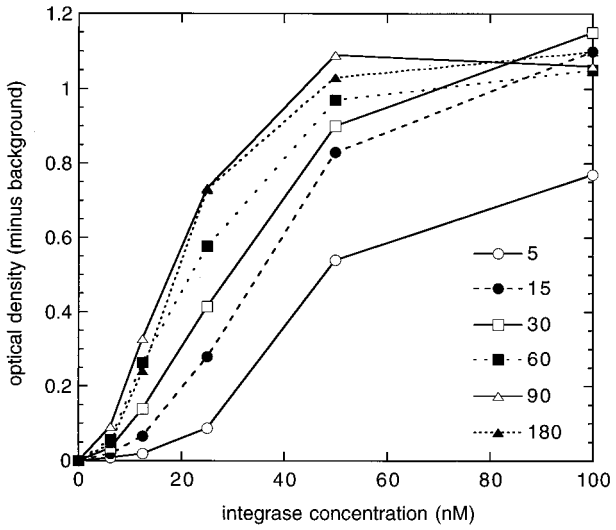

FIG. 8. Time and protein concentration dependence of the assembly reaction. Assembly reactions were performed at 37°C with integrase at concentrations of 5 to 100 nM. Each concentration of enzyme was incubated with the immobilized donor for 5, 15, 30, 60, 90, or 180 min. At each time, the unassociated enzyme was removed as in previous experiments, and strand transfer activity of the resultant complex was assessed by adding the target substrate and measuring activity in a standard 30-min reaction.

DISCUSSION

Given the apparent stability demonstrated for integration complexes assembled in vivo (3, 14, 36, 42), interfering with this step in the replication cycle of HIV may require inhibitors which can prevent the assembly process and/or irreversibly disrupt the stable association between integrase and the viral DNA. Therefore, particularly with respect to potential inhibitors, the ability to analyze the integrase-donor substrate interaction as an in vitro-assembled strand transfer complex may prove to be invaluable in the development of effective antiviral agents directed against this therapeutic target. In this communication, we have characterized the in vitro interaction between HIV-1 integrase and the viral donor DNA substrate as a function of strand transfer. We have used immobilized LTR oligonucleotides to uncouple various steps in the strand transfer reaction and have shown this method to be useful in the study of both the assembly and the dynamics of a productive integrase-donor substrate complex.

Our results demonstrate that (i) a distinct divalent cation requirement is exhibited by the assembly and strand transfer reactions, (ii) the requirement for high concentrations of $MnCl_2$ in vitro is specific for the assembly reaction and correlates with promoting the multimerization of integrase irrespective of the presence of substrate, (iii) preincubation of the enzyme in high concentrations of manganese circumvents the requirement for superphysiological concentrations of manganese in the reaction, (iv) integrase assembles on the immobilized donor DNA as a stable multimer wherein more than one functional active site contributes to strand transfer activity, and (v) as assembled on the immobilized donor, this multimeric complex is sufficient to catalyze the entire reaction, 3′-end processing as well as strand transfer. In vitro as in vivo, the enzymatic complex assembles on the LTR donor substrate prior to interacting with the second substrate, the target DNA (15, 40).

The enzymatic functions of integrase, 3′-end processing as well as strand transfer, are accomplished by a multimeric form

of the protein (16, 17, 26, 37). However, integrase which is monomeric in solution is catalytically active (21), and therefore multimerization may occur upon substrate binding. Consistent with the latter interpretation, we observed that assembly of the strand transfer complex on the immobilized LTR was both time and protein concentration dependent. In addition, integrase subunits could be readily exchanged during assembly. Although mutations in distinct domains of integrase can complement enzymatic function in vitro (16, 17, 26), we have shown that multimeric complexes in which a proportion of the subunits contain an active-site lesion are functionally impaired relative to the wild-type complex. These results suggest that the catalytic sites from more than one subunit in the multimer may contribute to the strand transfer reaction. The data support results of mutagenesis studies which have shown that the active sites for the 3′-end processing reaction and catalysis of strand transfer are functionally nondistinct, implying that individual subunits may be required for the 3′-end processing and transfer steps in the reaction.

Optimal strand transfer activity was achieved in reactions with at least four molecules of integrase per donor substrate; therefore, integrase may bind and catalyze strand transfer as a tetramer. Although the concentration required to achieve maximal activity may reflect the concentration of integrase necessary to promote the necessary multimerization, tetramers, as well as dimers, of integrase have been observed in solution for both the full-length HIV and Rous sarcoma virus integrases (22, 26, 39). In contrast to the full-length protein, truncated proteins encompassing the catalytic domain (amino acids 50 to 212) associate in solution as monodisperse dimers (13). Although functional in the disintegration reaction (13), the truncated catalytic domain protein is inactive in both the 3′-end processing and strand transfer reactions. The truncated enzyme lacks both the C-terminal DNA binding domain and the N-terminal zinc finger domain of integrase.

Manganese or magnesium is required for assembly of the stable integrase-donor substrate complex (15, 40) and catalysis (35, 39), and both have been shown to promote aggregation of the enzyme, both in the presence and in the absence of DNA (16, 24). We have demonstrated a functional distinction between the divalent cation requirements for catalysis and assembly and have shown that the latter corresponds to the ability of the metal to induce a more active, multimeric form of the enzyme in solution. In vitro, assembly of the enzymatically competent complex on the immobilized donor substrate required high concentrations of divalent cation, with the optimal concentration for $MnCl_2$ determined to be 25 mM. In contrast, after assembly, the strand transfer reaction was efficiently catalyzed at concentrations of $MnCl_2$ as low as 1 mM. Although the presence of high concentrations of $MnCl_2$ was not critical to maintain the complex in an enzymatically active form, preassembled complexes were sensitive to EDTA, suggesting that loosely associated manganese was also required for stable binding to the donor DNA substrate.

Concentrations of $MnCl_2$ which supported assembly on the immobilized donor substrate were also shown to promote the multimerization of integrase in the absence of a DNA substrate. Preincubation of the enzyme in 25 mM $MnCl_2$ induced multimerization and alleviated the requirement for high concentrations of divalent cation during assembly. Therefore, the superphysiological requirement for the $MnCl_2$ in vitro may in part be explained by an apparent deficiency in the ability of the recombinantly expressed protein to assume the appropriate multimeric form.

The N terminus of integrase contains a highly conserved zinc finger motif (4) which has been shown to be important in promoting higher-order protein-protein interactions and has also been implicated in mediating the stable association with the donor DNA (16, 24). It is therefore possible that the high concentrations of manganese which are required for multimerization and assembly are utilized by the enzyme as a surrogate for zinc in this critical structural motif. In this regard, we have observed that compared with manganese, lower concentrations of zinc induce integrase multimerization (23a). However, zinc is a potent inhibitor of the strand transfer reaction, and we have not been able to assess the effect of the zinc-induced multimerization on strand transfer directly (23a). The presence of zinc in integrase has not been explicitly demonstrated; however, previous studies have shown that peptides which encompass the putative zinc finger motif bind zinc stoichiometrically (4). Given that this association could be readily disrupted (4), it is not unlikely that during purification of the recombinant protein, the endogenous metal may be removed resulting in an apoenzyme which is catalytically less active than the potentially metal-bound form.

Once the active multimeric complex has assembled on the immobilized donor substrate, the subunits do not readily dissociate, as evidenced by the fact that the strand transfer activity of the preassembled complex was unperturbed by challenge with inactive subunits. This experimental result suggests that integrase mutants expressed as potential transdominant negatives are not likely to express a transdominant negative phenotype or interfere with the function of HIV integrase in vivo. In vitro, the complex was also stable to concentrations of salt and mild chaotropes which abolish assembly and catalysis (15, 23a), and neither the stability nor the avidity of the complex was affected by preprocessing of the 3′ end. In agreement with recently published studies on the interaction between integrase and LTR substrates in solution (15), our results suggest that strand transfer complexes assembled in vitro reconstitute certain biochemical properties attributed to preintegration complexes isolated from HIV-infected cells. Therefore, these studies extend and substantiate the suitability of this experimental method for the isolation and mechanistic analysis of potentially biologically interesting inhibitors of HIV-1 integrase.

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