

Differential Divalent Cation Requirements Uncouple the Assembly and Catalytic Reactions of Human Immunodeficiency Virus Type 1 Integrase

DARIA J. HAZUDA,* PETER J. FELOCK, JEFFREY C. HASTINGS, BERNALI PRAMANIK,
AND ABIGAIL L. WOLFE

Department of Antiviral Research, Merck Research Laboratories, West Point, Pennsylvania 19486

Received 4 March 1997/Accepted 1 June 1997

Previous in vitro analyses have shown that the human immunodeficiency virus type 1 (HIV-1) integrase uses either manganese or magnesium to assemble as a stable complex on the donor substrate and to catalyze strand transfer. We now demonstrate that subsequent to assembly, catalysis of both 3' end processing and strand transfer requires a divalent cation cofactor and that the divalent cation requirements for assembly and catalysis can be functionally distinguished based on the ability to utilize calcium and cobalt, respectively. The different divalent cation requirements manifest by these processes are exploited to uncouple assembly and catalysis, thus staging the reaction. Staged 3' end processing and strand transfer assays are then used in conjunction with exonuclease III protection analysis to investigate the effects of integrase inhibitors on each step in the reaction. Analysis of a series of related inhibitors demonstrates that these types of compounds affect assembly and not either catalytic process, therefore reconciling the apparent disparate results obtained for such inhibitors in assays using isolated preintegration complexes. These studies provide evidence for a distinct role of the divalent cation cofactor in assembly and catalysis and have implications for both the identification and characterization of integrase inhibitors.

The human immunodeficiency virus (HIV) integrase represents a potential target for the development of selective anti-retrovirus chemotherapeutic agents. Integration is an essential and defining step in the replication of all retroviruses, including HIV (for a review, see references 2, 17, and 36). The virally encoded enzyme integrase which catalyzes the sequence-specific 3' end processing and DNA strand transfer reactions that constitute this stepwise process is the only protein known to be essential for integration (22).

Integrase is packaged together with the viral reverse transcriptase and the positive-sense RNA genome into HIV particles (18). Following reverse transcription, the double-stranded viral DNA is maintained as a stable nucleoprotein complex consisting of integrase as well as a number of additional viral and cellular components (3, 13, 21, 35, 40). In the context of this nucleoprotein complex, integrase specifically associates with sequences within the long terminal repeat (LTR) regions and processes each 3' end of the linear viral genome. Removal of the 3'-terminal dinucleotide generates two recessed ends, which then serve as the donor substrates in strand transfer. In the strand transfer reaction, integrase nicks the cellular DNA and transfers each recessed 3' viral end onto the 5' ends of the nicked target substrate.

In vivo and in vitro, the strand transfer reaction can be temporally and spatially segregated from binding and processing of the viral DNA. In vivo, HIV nucleoprotein complexes may persist as stable preintegrative intermediates (35); when isolated from infected cells, these preintegration complexes can be used to perform integration reactions in vitro (14). In vitro, in the absence of a target DNA substrate, the recombinant enzyme will form stable complexes with HIV LTR sequence oligonucleotides (11, 38, 39). Assembly of a stable

integrase donor substrate complex and strand transfer are divalent cation-dependent processes requiring either manganese or magnesium (11, 38, 39).

In vitro systems which use recombinantly expressed integrase have been developed for biochemical analysis as well as to screen for and characterize potential inhibitors (1, 4, 6, 20, 34). By using such assay systems, a variety of structurally related integrase inhibitors have been identified (5, 7–10, 15, 16, 23, 25–29, 32). Although these types of inhibitors have been shown to be equally effective against virally derived integrases in either half-site (single-donor) or concerted, full-site (two-donor) strand transfer reactions (19), many such compounds are inactive in reactions catalyzed by preintegration complexes isolated from HIV-infected cells (14).

In this study, we used a variety of divalent cations to investigate the cofactor requirement for assembly and catalysis, respectively. We present evidence suggesting a distinct role for the divalent cation in assembly and 3' end processing or strand transfer. The unique divalent cation requirements for assembly and catalysis are used to stage the reaction and analyze a series of previously described integrase inhibitors with respect to each of these processes. The integrase inhibitors examined in this study are shown to prevent assembly and have no effect on either of the subsequent catalytic reactions. These results are consistent with the failure of such compounds to affect in vitro integration reactions catalyzed by stable preintegration complexes isolated from HIV-infected cells (14). The implications for identifying compounds which may inhibit the activity of stable preintegration complexes and restrict the replication of HIV are discussed.

MATERIALS AND METHODS

Oligonucleotide DNA substrates and recombinant HIV-1 integrase. The oligonucleotides representing the HIV type 1 (HIV-1) HXB2 U5 LTR sequence used as the 3' end processing substrate, the U5 LTR oligonucleotides used to coat microtiter plates (Covalink; NUNC), and the biotinylated oligonucleotide used as the target DNA substrate were synthesized by Midland Certified Reagent

* Corresponding author. Mailing address: Department of Antiviral Research WP 16-100, Merck Research Laboratories, West Point, PA 19486. Phone: (215) 652-7918. Fax: (215) 652-0994.

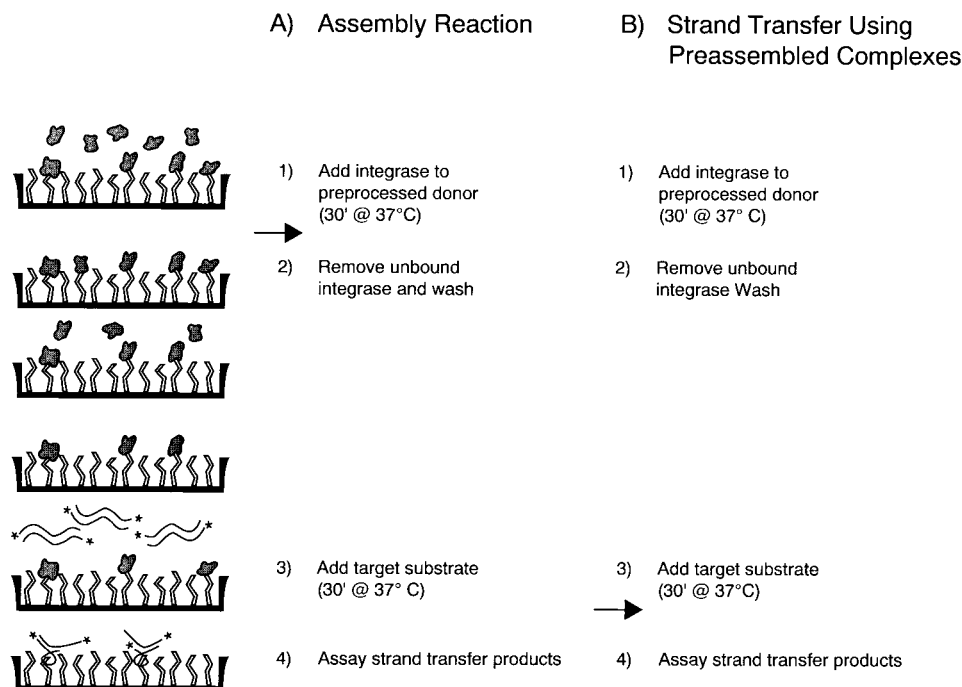


FIG. 1. Schematic of staged microtiter strand transfer assays. (A) The effects of cations or inhibitors on assembly are assessed using preprocessed donor substrates. The divalent cation (25 mM CaCl_2 , CoCl_2 , or MnCl_2) or inhibitor is varied at the time of donor binding. After 30 min (30'), the buffer is removed along with any unassociated enzyme. The strand transfer reaction is performed in buffer containing 25 mM MnCl_2 in the absence of inhibitor. (B) The effect of divalent cations or inhibitors on strand transfer is analyzed by using preassembled complexes. Complexes are assembled using preprocessed donor substrates. After unassociated enzyme is removed, divalent cation (25 mM CaCl_2 , CoCl_2 , or MnCl_2) or inhibitor is added, followed immediately by the biotinylated target substrate. The strand transfer reaction is performed in the presence of the inhibitor.

Co. (Midland, Tex.). Expression and purification of the recombinant HIV-1 HXB2 integrase in *Escherichia coli* was reported earlier (20).

Assay for 3' end processing. The assay for specific 3' end processing of LTR donor sequence oligonucleotide substrates was performed essentially as described previously (23) except that 250 nM integrase was used and 25 mM MgCl_2 , MnCl_2 , CoCl_2 , and/or CaCl_2 was added as indicated. All reactions were performed in a final concentration of 10% dimethyl sulfoxide (DMSO). The N-2-specific 3' end processing products were detected following electrophoresis on 20% sequencing gels.

Microtiter plate assay for strand transfer. The U5 LTR sequence oligonucleotides were synthesized to include a 5' phosphate on the DNA strand which is processed by integrase. The presence of the phosphate group exclusively on the 5' end of the processed strand ensures that the LTR substrate oligonucleotide is immobilized with the appropriate orientation (20).

The microtiter plate assay for strand transfer was performed with an immobilized 30-bp U5 donor substrate and a 20-bp target substrate biotinylated at the 3' end of each DNA strand (20, 39). Unless indicated, each reaction mixture of 100 μl included 20 mM HEPES (pH 7.8), 25 mM NaCl, 25 mM MnCl_2 , 5 mM β -mercaptoethanol, 50 μg of bovine serum albumin per ml and integrase (50 nM, final concentration). All reactions were performed in 10% DMSO. Integrase was assembled with immobilized LTR oligonucleotides for 30 min at 37°C. For coupled assays, the strand transfer reaction was initiated with the addition of 5 μl of the biotinylated target substrate (150 nM, final concentration). For other assay configurations, unbound enzyme was removed and the reaction buffer was exchanged before addition of the target substrate (39) (Fig. 1). Strand transfer reaction mixtures were incubated for 30 min at 37°C. Strand transfer products were detected by using alkaline phosphatase-conjugated avidin (Boehringer Mannheim, Indianapolis, Ind.).

Fifty percent inhibitory concentrations (IC_{50} s) for inhibitors were estimated from titration series of twofold dilutions of each compound starting at 100 μM . For coupled reactions, compounds were added immediately prior to the addition of integrase; after 30 min, the target DNA substrate was added without any wash or buffer exchange, and the reaction mixture was incubated for an additional 30 min at 37°C. For assembly (Fig. 1A), compounds were added together with integrase; after the initial 30-min incubation, the reaction mix was removed, the wells were washed three times, and the strand transfer reaction was performed in the absence of inhibitor. For strand transfer (Fig. 1B), complexes were preassembled for 30 min in the absence of inhibitor; the reaction mix was removed, and the complexes were washed as before. The reaction buffer was replaced, and the compounds were added immediately prior to the addition of the target DNA.

Percent inhibitory activity was calculated relative to the appropriate control reaction performed in 10% DMSO. For the assembly and strand transfer assays, precleaved donor substrates were used.

Exonuclease III protection analysis. Immobilized LTR donor substrate oligonucleotides were radiolabeled by using T4 polynucleotide kinase. For a 96-well microtiter plate, 70 μl of the following reaction mixture was added to each well: 40 μl of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 mCi/ml, >5,000 Ci/mmol; Amersham) and 40 μl of T4 polynucleotide kinase (Boehringer Mannheim) in 7 ml of reaction buffer supplied by the manufacturer. Reaction mixtures were incubated at 37°C for 1 h. The kinase reaction mixture was removed, and the plates were washed twice with integrase reaction buffer (see above) without divalent cation. Since the 5' end of the DNA strand which is endonucleolytically processed by integrase is covalently linked to the plate surface, the reaction specifically labels the 5' end of the nonprocessed DNA strand.

Radiolabeled immobilized donor substrates were used in exonuclease III protection studies. Integrase (250 nM) was incubated with the donor substrate for 30 min at 37°C in the presence or absence of divalent cation or inhibitor. Excess enzyme was removed; complexes were washed once with reaction buffer containing 0.5 M NaCl and then twice in reaction buffer with 50 mM NaCl. Exonuclease III was added at 100 μl /well (500 U of exonuclease III per ml in buffer supplied by the manufacturer (Promega). Digestion was allowed to proceed for 5 min at room temperature. The exonuclease was removed, and the reactions were terminated by adding 50 μl of sequencing gel loading buffer to each well. Samples were heated to 95°C and analyzed on 20% sequencing gels.

RESULTS

Distinct divalent cations promote assembly and strand transfer. In vitro and in vivo, assembly of a stable complex between a multimer of integrase and the viral DNA donor substrate initiates the multistep process of integration: sequence-specific endonucleolytic processing of the 3' end of the viral donor DNA substrate followed by strand transfer of the donor substrate into the double-stranded target DNA (11, 35, 38, 39). The divalent cations manganese and magnesium have been shown to facilitate multimerization and assembly of integrase and are required in the catalysis of strand transfer (11,

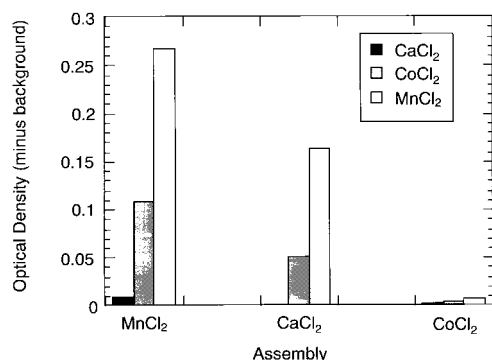


FIG. 2. Effects of calcium, cobalt, and manganese on assembly and strand transfer. The assembly and strand transfer reactions were performed as described in Materials and Methods, using preprocessed LTR donor substrates. Complexes were assembled for 30 min, using either 25 mM CaCl₂, CoCl₂, or MnCl₂ as indicated. The reaction mixture was then removed; the complexes were washed with buffer containing no divalent cation, and the buffer was then replaced with reaction buffer containing CaCl₂, CoCl₂, or MnCl₂. The strand transfer reaction was initiated by the addition of the biotinylated target substrate.

12, 24, 31, 37–39). The structurally related bacterial transposase MuA also assembles as an obligate multimer on its respective donor substrate(s) (reviewed in reference 33). MuA requires either manganese or magnesium for catalysis but will use calcium as well as manganese or magnesium for assembly (30).

The effects of divalent cations other than magnesium or manganese on the various reactions mediated by HIV-1 integrase have not been heretofore examined. In the following experiments, immobilized LTR sequence donor oligonucleotides were used to evaluate the efficacy of calcium, cobalt, or manganese in assembly and strand transfer as outlined in Fig. 1. Integrase has previously been shown to assemble as a stable complex on immobilized donor substrate oligonucleotides; these substrates can therefore be used to uncouple assembly and strand transfer and characterize both steps in the reaction (39). To obviate the requirement for 3' end processing, assays were performed with preprocessed donors, i.e., oligonucleotides lacking the terminal 3' dinucleotide.

Integrase was incubated with immobilized donor oligonucleotides in the presence of calcium, cobalt, or manganese (Fig. 2). Enzyme not stably associated with the donor substrate was removed, and the complexes were washed extensively to eliminate the potential of contaminating divalent cation. For each of the three assembly conditions, the strand transfer reaction was then performed in either calcium, cobalt, or manganese. The buffer was replaced with buffer containing the appropriate divalent cation, and strand transfer was initiated by the addition of the biotinylated target DNA. The reaction mixtures were incubated for 30 min, and the biotinylated strand transfer products were quantified.

As shown in Fig. 2, when calcium or manganese was included during assembly, strand transfer products were observed when the latter reaction was performed with cobalt or manganese but not calcium; therefore, assembly of an enzymatically competent complex was promoted by both calcium and manganese, although calcium could not substitute in strand transfer. Cobalt effectively promoted the strand transfer reaction catalyzed by complexes assembled in either calcium or manganese; however, when cobalt was substituted during assembly, reaction products were not generated irrespective of which divalent cation was included during strand transfer. The observation that the reaction occurred when calcium was used

during assembly and then exchanged for cobalt during strand transfer but not when the order was reversed (Fig. 2) demonstrates that the divalent cation requirements for assembly and strand transfer can be functionally discriminated based on the ability to substitute calcium and cobalt, respectively. The ability to use unique divalent cations to promote assembly and strand transfer indicates a distinct role for the cofactor in each of these processes.

The 3' end processing reaction requires divalent cations for both assembly and catalysis. The divalent cation requirement for assembly is functionally distinct from the enzymatic cofactor requirement in strand transfer, suggesting that 3' end processing may also exhibit separate divalent cation requirements for assembly and catalysis. Since manganese and magnesium are used to analyze 3' end processing and these divalent cations promote both assembly and catalysis (12, 31, 37, 39), the catalytic cofactor requirement for the endonucleolytic reaction has not been previously addressed.

To investigate the divalent cation requirements in 3' end processing, integrase was incubated with a radiolabeled 20-bp donor substrate oligonucleotide in the absence of divalent cation (Fig. 3A) or in the presence of calcium (Fig. 3B), cobalt (Fig. 3C), magnesium (Fig. 3D), or manganese (Fig. 3E) for 30 min at 37°C, conditions in which calcium, manganese, and magnesium support assembly of integrase on immobilized donor substrates (Fig. 2 and reference 20a). For each preincubation condition, buffer with no divalent cation or with 25 mM calcium, cobalt, magnesium, or manganese was then added (Fig. 3). The reaction mixtures were incubated for an additional 30 min, and the products were analyzed after electrophoresis on sequencing gels.

As shown in Fig. 3A, when divalent cation was omitted during the preincubation, generation of the characteristic N-2, 18-nucleotide 3' endonucleolytic processing product was not observed unless either magnesium or manganese was included during the second incubation (lanes 4 and 5). The addition of calcium had no effect (lane 2), and cobalt elicited marginal 3' processing activity (lane 3). In contrast, when the preincubation was performed in calcium, the 3' end processing reaction was equally efficient when cobalt, magnesium, and manganese were added for the subsequent incubation (Fig. 3B, lanes 3, 4, and 5, respectively). Although neither calcium nor cobalt alone substituted for magnesium or manganese in the 3' end processing reaction (compare lanes 1 in Fig. 3B and C with lanes 1 in Fig. 3D and E), when integrase was assembled with the donor substrate in the presence of calcium, cobalt promoted the subsequent endonucleolytic reaction (Fig. 3B, lane 3). Therefore, as was observed in the strand transfer assay, 3' processing requires the divalent cation for both assembly and catalysis, and the divalent cation requirements for assembly and catalysis can be distinguished based on the ability to use either calcium or cobalt. These results also demonstrate that calcium can be used to stage the reaction in solution and functionally uncouple endonucleolytic processing and strand transfer from assembly.

Integrase inhibitors have no effect on catalysis subsequent to assembly. The availability of biochemical assay systems and recombinant integrase has facilitated the discovery of integrase inhibitors which are effective in reactions catalyzed by the recombinant enzyme in vitro. Many compounds described as inhibitors of integrase have in common the presence of one or more hydroxyl substituents; for these inhibitors, the number and arrangement of hydroxyl groups are critical (9, 10, 15, 16, 23, 27, 28, 32). Representative inhibitors included in this class are listed in Table 1. Although a subset of these compounds inhibit both half-site and full-site concerted reactions mediated

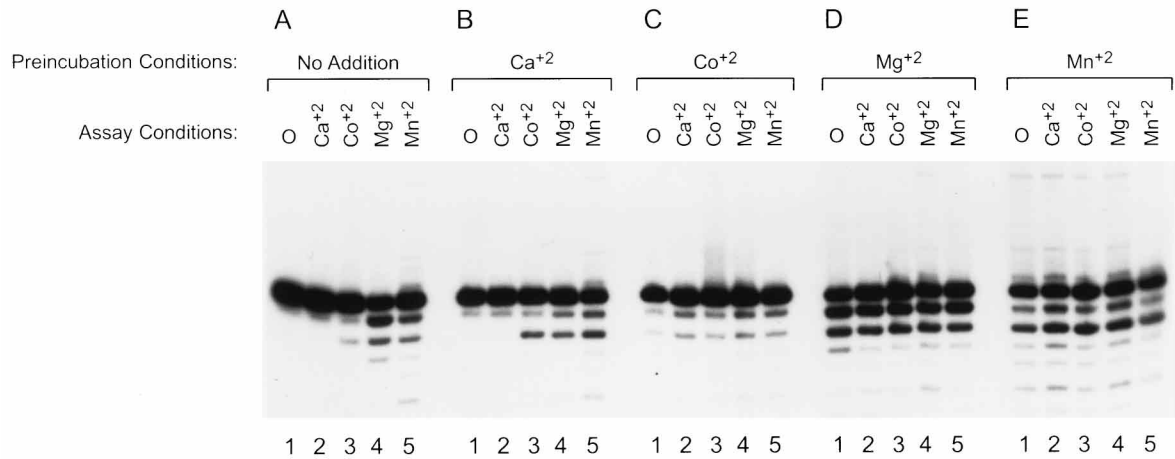


FIG. 3. Effects of divalent cations on assembly and 3' end processing. Integrase was preincubated with the radiolabeled 20-bp U5 LTR oligonucleotide substrate in the presence of no divalent cation (A) or 25 mM CaCl₂ (B), CoCl₂ (C), MgCl₂ (D), or MnCl₂ (E). After 30 min at 37°C, CaCl₂, CoCl₂, MgCl₂, or MnCl₂ (5 μl of a 0.25 M preparation) was added to each 50-μl reaction as indicated above lanes 2 to 5. Mixtures were then incubated for 30 min at 37°C and analyzed on 20% sequencing gels.

by integrase isolated from HIV virions (19), many exhibit no activity in in vitro integration reactions catalyzed by preintegration complexes isolated from HIV-1-infected cells (14). Although these results suggest that the integration reaction catalyzed by preintegration complexes may be intrinsically less sensitive to inhibition, it is also possible that such inhibitors prevent assembly and have little or no effect on subsequent catalysis. The ability to uncouple each of the catalytic reactions from assembly makes it feasible to address the latter possibility directly.

To evaluate inhibition of 3' end processing subsequent to and independent of assembly, the reaction was staged in solution by using calcium as outlined in Fig. 3. Integrase was preincubated with the radiolabeled donor substrate oligonucleotide in the presence of calcium; inhibitors were then added postassembly, and the catalytic reaction was initiated with manganese (Fig. 4B). The results from these staged assays are compared with the results from coupled reactions in which integrase was preincubated with the radiolabeled donor in the absence of divalent cation and the inhibitors were added followed immediately by manganese; in these reactions, the addition of the inhibitor precedes both assembly and catalysis, since neither can occur prior to addition of manganese (Fig.

4A). Finally, to differentiate potential effects on multimerization from inhibition of substrate binding, integrase was preincubated in manganese prior to the addition of the inhibitor and the radiolabeled donor substrate (Fig. 4C). Since manganese promotes the multimerization of integrase (31, 39), under these conditions the inhibitor is therefore introduced subsequent to multimerization but prior to assembly and catalysis.

As shown in Fig. 4, each compound (1 to 7 in Table 1) was inhibitory in the coupled 3' processing reaction, i.e., when added after the preincubation of integrase with the donor substrate in the absence of divalent cation (Fig. 4A). Under these conditions, neither multimerization or assembly occurs before the addition of the inhibitor. The inhibitors were also effective when added after the preincubation of integrase in manganese (Fig. 4C), i.e., conditions which promote multimerization. In contrast, these inhibitors were ineffective when added after assembly. No inhibition of 3' end processing was observed when the compounds were added after preincubation of integrase with the donor substrate in the presence of calcium (Fig. 4B). The results in Fig. 4A and B demonstrate that these inhibitors must be present during assembly to be efficacious. In addition, the results in Fig. 4C suggest that inhibition is independent of and subsequent to multimerization.

To confirm these observations, we performed analogous studies relative to assembly and strand transfer, using immobilized substrates in the microtiter plate strand transfer assay outlined in Fig. 1. For assembly (Fig. 1A), integrase was incubated with the preprocessed donor substrates in the presence of the inhibitor (1 to 100 μM). After 30 min, the inhibitor along with any unassociated enzyme was removed, and the strand transfer reaction was initiated by the addition of the biotinylated target DNA substrate. The strand transfer reaction was then performed in the absence of inhibitor. For strand transfer (Fig. 1B), complexes were first assembled in the absence of inhibitor. Excess integrase was removed, and the compounds were added immediately prior to the addition of the target DNA substrate. The strand transfer reaction was performed in the presence of the inhibitor, and the reaction products were quantified as described above. The results of the staged assembly and strand transfer assays are compared with results from coupled reactions wherein the inhibitor was added

TABLE 1. Inhibition of coupled and staged strand transfer assays				
Compound		IC ₅₀ (μM)		
No.	Name (reference[s])	Coupled ^a	Assembly ^b	Preassembled ^c
1	Quercetagenin (16, 32)	0.75	0.75	25
2	Hematoxylin (23)	1	2	100
3	Bistyrphostin (28)	0.7	2	45
4	β-Conidendrol (23)	1	5	>100
5	Hematein	0.75	0.75	35
6	Aurin tricarboxylic acid (9, 10)	0.7	1	10
7	Dihydroxynaphthoquinone (15)	10	15	100

^a Compounds were added with the addition of integrase and were present throughout the assay.
^b Compounds were added during assembly and removed prior to addition of the target substrate (Fig. 1A).
^c Complexes were preassembled and compounds were added along with the target substrate (Fig. 1B).

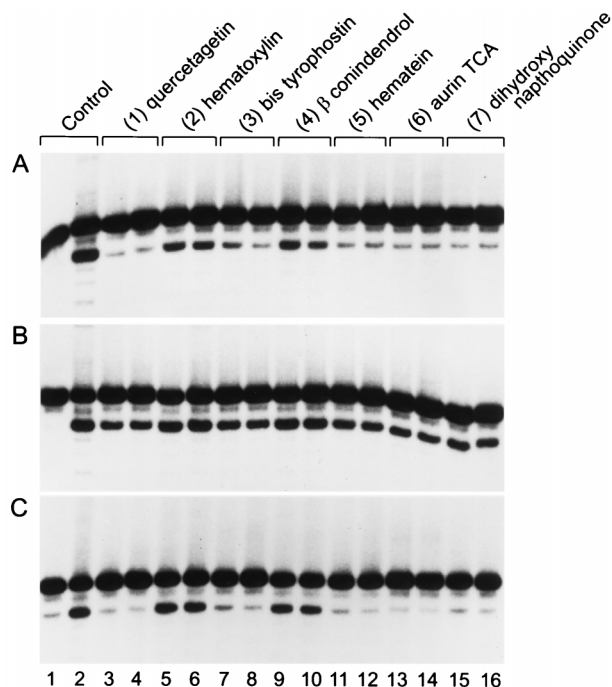


FIG. 4. Effects of inhibitors on staged 3' end processing reactions. Integrase was preincubated with the radiolabeled 20-bp U5 LTR oligonucleotide substrate in the absence or presence of 25 mM CaCl_2 (A or B, respectively) or 25 mM MnCl_2 in the absence of radiolabeled donor substrate (C). All preincubations were performed in a final volume of 47.5 μl (20 mM Tris-HCl [pH 7.8], 25 mM NaCl, 5 mM β -mercaptoethanol, 5% DMSO). After 30 min at 37°C, 2.5 μl of each compound was then added such that the final concentration was either 10 or 20 μM . In panels A and B, the 3' end processing reaction was initiated by the addition of 5 μl of 250 mM MnCl_2 (25 mM, final concentration). In panel C, reactions were initiated with the addition of radiolabeled donor substrate. The 3' end processing reaction mixtures were incubated for 30 min at 37°C and analyzed on 20% sequencing gels. For all three preincubation conditions, control reactions were performed in 10% DMSO without inhibitor in the absence or presence of manganese (lanes 1 and 2, respectively). TCA, tricarboxylic acid.

during assembly and not removed (Table 1). The latter format is comparable to the coupled solution assays of other laboratories, and the relative activity determined for these inhibitors in this reaction is consistent with data previously published.

As shown in Table 1, the relative potency of each inhibitor in assembly was comparable to that observed in the coupled assay. Each compound effectively inhibited the assembly of a catalytically active strand transfer complex between integrase and the immobilized donor oligonucleotide substrate with an IC_{50} of 1 μM or less. In contrast, the compounds were 10- to 100-fold less potent in strand transfer when added after assembly. The strand transfer data in Table 1 and the 3' end processing results in Fig. 4 demonstrate that these inhibitors have little or no effect on either of the catalytic reactions mediated by stable integrase-donor substrate complexes. In both sets of experiments, the inhibitors were effective only when present at the time of assembly.

Integrase inhibitors prevent the interaction between integrase and the donor substrate. The previous results are consistent with a mechanism in which the inhibitor prevents assembly of a stable, enzymatically competent complex between integrase and the donor substrate; however, the lack of activity observed in strand transfer indicates no effect on binding to the nonspecific target DNA substrate. The productive interaction of integrase with LTR donor substrates is functionally distinct from nonspecific DNA binding. Although integrase will bind

DNA nonspecifically in the absence of divalent cation, assembly of stable complex with LTR donor substrates requires divalent cation (11, 38, 39).

The ability of integrase to protect the LTR sequence oligonucleotides from digestion by exonuclease(s) has previously been used to demonstrate a physical association between integrase and donor DNA substrates in solution (31). A similar strategy using exonuclease III was developed to study the effect of inhibitors on the association between integrase and immobilized donor substrates. Immobilized oligonucleotides were radiolabeled directly on a microtiter plate by using T4 polynucleotide kinase; since the processed strand was covalently linked onto the plate surface via the 5' end, the radiolabel is incorporated exclusively on the 5' end of the nonprocessed strand and therefore proximal to the sequences required for 3' end processing and strand transfer.

To validate the use of exonuclease III to detect stable complexes, divalent cation dependence was first investigated. Integrase was incubated with the labeled donor substrates in the presence or absence of divalent cation. The complexes were washed once with 0.5 M NaCl to reduce and/or eliminate nonspecific binding. (This treatment has no effect on catalytic activity [data not shown].) Complexes were then treated briefly with exonuclease III. As shown in Fig. 5A, when manganese, calcium, and to a lesser extent magnesium were included during assembly (lanes 7 and 8, 9 and 10, and 11 and 12, respectively), the amount of substrate resistant to exonuclease III digestion was substantially increased compared to either the no-integrase (lanes 3 and 4) or no-divalent cation (lanes 5 and 6) control reaction. The observation that exonuclease protection was observed only when divalent cations were included in the reaction (compare lanes 5 and 6 with lanes 7 to 12) indicates the analysis reflects the assembly of a stable complex. These results also confirm the observation that calcium can replace manganese and magnesium in assembly.

Exonuclease III protection was then used to assess whether inhibitors compromise the assembly of a stable integrase donor substrate complex. Complexes were assembled in the presence of inhibitor (compounds 1 to 7 at 25 μM), using manganese as in the previous experiment, and then subjected to exonuclease III digestion. As shown in Fig. 5B, each of the inhibitors (lanes 3 to 9) significantly decreased the amount of donor substrate protected from exonuclease digestion relative to the DMSO integrase control reaction (lane 2); for most compounds, the level of substrate was reduced to that of the no-integrase control (lane 1). Compounds 1, 3, 5, 6, and 7, which were most effective in the previous analysis of 3' end processing, were also the most effective in abolishing exonuclease III protection; compounds 2 and 4, which were moderately active in the previous experiment, were also moderately active in the exonuclease III protection analysis (compare Fig. 4A and C with Fig. 5B). The exonuclease III protection data are therefore consistent with the results of the 3' processing and strand transfer experiments in Table 1 and Fig. 4. In addition, these results provide direct evidence that the inhibitors prevent assembly of a stable complex between integrase and the LTR donor substrate and provide a mechanistic basis for the observed inhibition.

DISCUSSION

In this study, we have dissected the assembly, 3' end processing, and strand transfer reactions in order to investigate the divalent cation cofactor requirement of HIV-1 integrase and the mechanism of integrase inhibitors *in vitro*. We found that (i) functionally distinct divalent cation requirements are

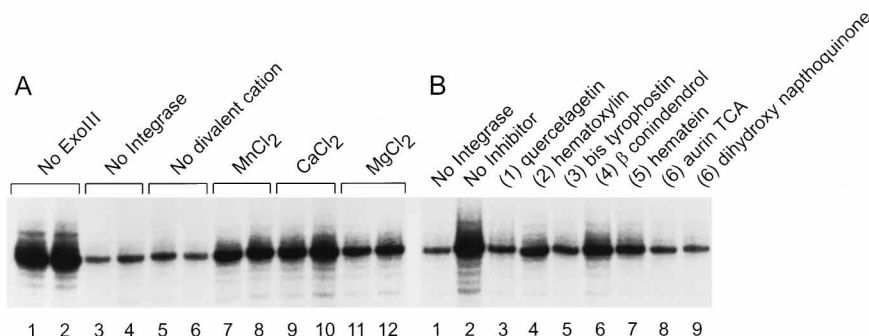


FIG. 5. Exonuclease III protection analysis of stable complex formation. LTR donor substrates immobilized in the wells of microtiter plates were radiolabeled on the 5' end of the nonprocessed strand as outlined in Materials and Methods. (A) Integrase was incubated for 30 min at 37°C with the labeled LTR donor substrate in the absence of divalent cation (lanes 5 and 6) or in the presence of 25 mM $MnCl_2$ (lanes 7 and 8), $CaCl_2$ (lanes 9 and 10), or $MgCl_2$ (lanes 11 and 12). Complexes assembled in the microtiter wells were washed once with 0.5 M NaCl and then twice in reaction buffer prior to treatment with exonuclease III (ExoIII) for 5 min at room temperature. The exonuclease reaction mixture was removed, loading buffer was added to the well, and the products were analyzed on 20% sequencing gels. Controls shown in lanes 1 and 2 were performed with integrase assembled in $MnCl_2$ without exonuclease III digestion. The exonuclease III control reactions in lanes 3 and 4 were performed in the absence of integrase. (B) Assembly was performed with 25 mM $MnCl_2$ as described in above and a final concentration of 25 μ M of each of the seven compounds indicated (lanes 5 to 9). The DMSO integrase control reaction is shown in lane 2.

manifest by assembly and catalysis in both the 3' end processing and strand transfer reactions, (ii) calcium will effectively substitute for the divalent cation requirement in assembly, whereas cobalt will mediate catalysis of both enzymatic reactions, (iii) the ability of calcium to substitute for manganese or magnesium in assembly allows functional uncoupling of the reaction in solution, (iv) using either calcium to stage the endonucleolytic reaction or immobilized donor substrates to uncouple the strand transfer reaction facilitates the mechanistic analysis of integrase inhibitors, and (v) in such uncoupled assay systems, structurally related inhibitors of integrase were shown to prevent the assembly of integrase as a catalytically active complex on the LTR donor substrate and to be ineffective inhibitors of either 3' end processing or strand transfer catalyzed by stable integrase donor substrate complexes assembled *in vitro*. This study therefore reconciles the discordant activities observed for such inhibitors in solution assays using either recombinant or virally derived integrase wherein assembly and catalysis are coupled (19) and assays using preintegration complexes isolated from HIV-infected cells in which the enzyme is preassembled on the viral substrate (14). The observation that integrase donor substrate complexes assembled *in vitro* by using the recombinant enzyme recapitulate the activity of HIV-1 preintegration complexes with respect to such inhibitors validates the utility of these assays for identifying and characterizing integrase inhibitors.

Despite relatively poor sequence homology, HIV-1 integrase and the bacterial transposase MuA are structurally homologous (reviewed in reference 44). Both the MuA transposase and integrase assemble as a stable, multimeric complex on the specific donor DNA substrate and catalyze similar one-step transesterification reactions (11, 30, 31, 38, 39). The observation that HIV-1 integrase, like MuA, will utilize calcium as well as magnesium or manganese for assembly extends the functional analogy between the two enzymes. The demonstration that unique divalent cations, i.e., calcium and cobalt, promote assembly and catalysis, respectively, indicates a distinct function for the divalent cation in assembly and both 3' processing and strand transfer. This observation is consistent with previous studies suggesting that the divalent cation requirement for assembly is separable from its function as an enzymatic cofactor in strand transfer (39). The ability to utilize cobalt as a cofactor for both 3' end processing and strand transfer sug-

gests functional and/or structural conservation in the two catalytic reactions.

Using calcium to promote assembly allows functional uncoupling of the reaction in solution. In conjunction with exonuclease III protection analysis, uncoupled solution reactions and staged assays using immobilized substrates were used to study a series of integrase inhibitors that are representative of many inhibitors of integrase which have been described to date (5, 7–10, 15, 16, 23, 25–29, 32). The inhibitors were shown to prevent the assembly of the catalytically active integrase-donor substrate complex and have no effect on the subsequent catalysis of either 3' end processing or strand transfer. These observations are consistent with previous studies which have shown that related compounds are inactive in reactions catalyzed by preintegration complexes isolated from HIV-infected cells (14). Incubation of integrase with substrate DNAs has also been shown to block labeling of the protein by 8-azido-ATP (25).

These results demonstrate that subsequent to assembly *in vitro* and in the course of infection *in vivo*, integrase becomes functionally resistant to many inhibitors which are routinely identified using assays in which assembly and catalysis are coupled. However, by adapting the methods developed for this analysis and configuring *in vitro* assays appropriately, it may be possible to identify novel classes of inhibitors which either maintain efficacy or preferentially interact with the assembled form of the enzyme. Given the stability which has been demonstrated for HIV-1 preintegration complexes *in vivo* (3), effective inhibition of integration may ultimately require inhibitors which retain activity subsequent to assembly. The characterization of integrase inhibitors by using assays such as those outlined in this study should therefore facilitate the identification and development of compounds which are efficacious as antiviral chemotherapeutic agents.

ACKNOWLEDGMENTS

We thank D. Grandgenett, R. LaFemina, S. Young, S. Singh, and E. Emini for helpful discussions, J. Cole for careful review of the manuscript, and D. Wilson for manuscript preparation.

REFERENCES

1. Billich, A., M. Schauer, S. Frank, B. Rosenwirth, and S. Billich. 1992. HIV-1 integrase: high-level production and screening assay for the endonucleolytic

- activity. *Antiviral Chem. Chemother.* **3**:113–119.
2. Brown, P. O. 1990. Integration of retroviral DNA. *Curr. Top. Microbiol. Immunol.* **157**:19–48.
 3. Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson. 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl. Acad. Sci. USA* **90**:6125–6129.
 4. Bushman, F. D., and R. Craigie. 1991. Activities of human immunodeficiency virus (HIV) integration protein *in vitro*: specific cleavage and integration of HIV DNA. *Proc. Natl. Acad. Sci. USA* **88**:1339–1343.
 5. Carteau, S., J. F. Mouscadet, H. Goulaouic, F. Subra, and C. Auclair. 1993. Inhibitory effect of the polyanionic drug Suramin on the *in vitro* HIV DNA integration reaction. *Arch. Biochem. Biophys.* **305**:606–610.
 6. Carteau, S., J. F. Mouscadet, H. Goulaouic, F. Subra, and C. Auclair. 1993. Quantitative *in vitro* assay for human immunodeficiency virus deoxyribonucleic acid integration. *Arch. Biochem. Biophys.* **300**:756–760.
 7. Carteau, S., J. F. Mouscadet, H. Goulaouic, F. Subra, and C. Auclair. 1993. Effect of topoisomerase inhibitors on the *in vitro* HIV DNA integration reaction. *Biochem. Biophys. Res. Commun.* **192**:1409–1414.
 8. Carteau, S., J. F. Mouscadet, H. Goulaouic, F. Subra, and C. Auclair. 1994. Inhibition of the *in vitro* integration of Moloney murine leukemia virus DNA by the DNA minor groove binder netropsin. *Biochem. Pharmacol.* **47**:1821–1826.
 9. Cushman, M., and P. Sherman. 1992. Inhibition of HIV-1 integration protein by aurointricarboxylic acid monomers, monomer analogs, and polymer fractions. *Biochem. Biophys. Res. Commun.* **185**:85–90.
 10. Cushman, M., W. M. Golebiewski, Y. Pommier, A. Mazumder, D. Reyman, E. De Clercq, L. Graham, and W. G. Rice. 1995. Cosalane analogues with enhanced potencies as inhibitors of HIV-1 protease and integrase. *J. Med. Chem.* **38**:443–452.
 11. Ellison, V., and P. O. Brown. 1994. A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration *in vitro*. *Proc. Natl. Acad. Sci. USA* **91**:7316–7320.
 12. Engelman, A., and R. Craigie. 1995. Efficient magnesium-dependent human immunodeficiency virus type 1 integrase activity. *J. Virol.* **69**:5908–5911.
 13. Farnet, C. M., and W. A. Haseltine. 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J. Virol.* **65**:1910–1915.
 14. Farnet, C. M., B. Wang, J. R. Lipford, and F. D. Bushman. 1996. Differential inhibition of HIV-1 preintegration complexes and purified integrase protein by small molecules. *Proc. Natl. Acad. Sci. USA* **93**:9743–9747.
 15. Fesen, M. R., K. W. Kohn, F. Leteurtre, and Y. Pommier. 1993. Inhibitors of human immunodeficiency virus integrase. *Proc. Natl. Acad. Sci. USA* **90**:2399–2403.
 16. Fesen, M. R., Y. Pommier, F. Leteurtre, S. Hiroguchi, J. Yung, and K. W. Kohn. 1994. Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. *Biochem. Pharmacol.* **48**:595–608.
 17. Goff, S. P. 1992. Genetics of retroviral integration. *Annu. Rev. Genet.* **26**:527–544.
 18. Grandgenett, D. P., A. C. Vora, and R. D. Schiff. 1978. A 32,000-dalton nucleic acid-binding protein from avian retrovirus cores possesses DNA endonuclease activity. *Virology* **89**:119–132.
 19. Hazuda, D., P. Felock, J. Hastings, B. Pramanik, A. Wolfe, G. Goodarzi, A. Vora, K. Brackmann, and D. Grandgenett. 1997. Equivalent inhibition of half-site and full-site retroviral strand transfer reactions by structurally diverse compounds. *J. Virol.* **71**:807–811.
 20. Hazuda, D. J., J. C. Hastings, A. L. Wolfe, and E. A. Emini. 1994. A novel assay for the DNA strand-transfer reaction of HIV-1 integrase. *Nucleic Acids Res.* **22**:1121–1122.
 - 20a. Hazuda, D., and A. L. Wolfe. Unpublished data.
 21. Karageorgos, L., P. Li, and C. Burrell. 1993. Characterization of HIV replication complexes early after cell-to-cell infection. *AIDS Res. Hum. Retroviruses* **9**:817–823.
 22. LaFemina, R. L., C. L. Schneider, H. L. Robbins, P. L. Callahan, K. LeGrow, E. Roth, W. A. Schleif, and E. A. Emini. 1992. Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. *J. Virol.* **66**:7414–7419.
 23. LaFemina, R. L., P. L. Graham, K. LeGrow, J. C. Hastings, A. Wolfe, S. D. Young, E. A. Emini, and D. J. Hazuda. 1995. Inhibition of human immunodeficiency virus integrase by bis-catechols. *Antimicrob. Agents Chemother.* **39**:320–324.
 24. Lee, S. P., H. G. Kim, M. L. Censullo, and M. K. Han. 1995. Characterization of Mg²⁺-dependent 3'-processing activity for human immunodeficiency virus type 1 integrase *in vitro*: real-time kinetic studies using fluorescence resonance energy transfer. *Biochemistry* **34**:10205–10214.
 25. Lipford, J. R., S. T. Worland, and C. M. Farnet. 1994. Nucleotide binding by the HIV-1 integrase protein *in vitro*. *J. Acquired Immun. Defic. Syndr.* **7**:1215–1223.
 26. Mazumder, A., D. Cooney, R. Agbaria, M. Gupta, and Y. Pommier. 1994. Inhibition of human immunodeficiency virus type 1 integrase by 3'-azido-3'-deoxythymidylate. *Proc. Natl. Acad. Sci. USA* **91**:5771–5775.
 27. Mazumder, A., K. Raghavan, J. Weinstein, K. W. Kohn, and Y. Pommier. 1995. Inhibition of human-immunodeficiency-virus type-1 integrase by curcumin. *Biochem. Pharmacol.* **49**:1165–1170.
 28. Mazumder, A., A. Gazit, A. Levitzki, M. Nicklaus, J. Yung, G. Kohlhausen, and Y. Pommier. 1995. Effects of tyrophostins, protein kinase inhibitors, on human immunodeficiency virus type 1 integrase. *Biochemistry* **34**:15111–15122.
 29. Mazumder, A., M. Gupta, D. M. Perrin, D. S. Sigman, M. Rabinovitz, M., and Y. Pommier. 1995. Inhibition of human immunodeficiency virus type 1 integrase by the phenanthroline-cuprous complex. *AIDS Res. Hum. Retroviruses* **11**:115–125.
 30. Mizuuchi, M., T. A. Baker, and K. Mizuuchi. 1992. Assembly of the active form of the transposase-Mu DNA complex: a critical control point in Mu transposition. *Cell* **70**:303–311.
 31. Pemberton, I. K., M. Buckle, and H. Buc. 1996. The metal ion-induced cooperative binding of HIV-1 integrase to DNA exhibits a marked preference for Mn(II) rather than Mg(II). *J. Biol. Chem.* **271**:1498–1506.
 32. Raghavan, K., J. K. Buolamwini, M. R. Fesen, Y. Pommier, K. W. Kohn, and J. N. Weinstein. 1995. Three-dimensional quantitative structure-activity relationship (QSAR) of HIV integrase inhibitors: a comparative molecular field analysis (CoMFA) study. *J. Med. Chem.* **38**:890–897.
 33. Rice, P., R. Craigie, and D. R. Davies. 1995. Retroviral integrases and their cousins. *Curr. Opin. Struct. Biol.* **6**:76–83.
 34. Sherman, P. A., and J. A. Fyfe. 1990. Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. *Proc. Natl. Acad. Sci. USA* **87**:5119–5123.
 35. Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* **9**:1551–1560.
 36. Varmus, H., and P. Brown. 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
 37. Vincent, K. A., V. Ellison, S. A. Chow, and P. O. Brown. 1993. Characterization of human immunodeficiency virus type 1 integrase expressed in *Escherichia coli* and analysis of variants with amino-terminal mutations. *J. Virol.* **67**:425–437.
 38. Vink, C., R. A. P. Lutzke, and R. H. A. Plasterk. 1994. Formation of a stable complex between the human immunodeficiency virus integrase protein and viral DNA. *Nucleic Acids Res.* **22**:4103–4110.
 39. Wolfe, A. L., P. J. Felock, J. C. Hastings, C. U. Blau, and D. J. Hazuda. 1996. 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. *J. Virol.* **70**:1424–1432.
 40. Zack, J. A., S. J. Arrigo, S. R. Weltsman, A. S. Go, A. Halslip, and I. S. Y. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213–222.