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# Recombinant Human Immunodeficiency Virus Type 1 Integrase Exhibits a Capacity for Full-Site Integration In Vitro That Is Comparable to That of Purified Preintegration Complexes from Virus-Infected Cells

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**Retrovirus preintegration complexes (PIC) in virus-infected cells contain the linear viral DNA genome (~10 kbp), viral proteins including integrase (IN), and cellular proteins. After transport of the PIC into the nucleus, IN catalyzes the concerted insertion of the two viral DNA ends into the host chromosome. This successful insertion process is termed "full-site integration." Reconstitution of nucleoprotein complexes using recombinant human immunodeficiency virus type 1 (HIV-1) IN and model viral DNA donor substrates (~0.30 to 0.48 kbp in length) that are capable of catalyzing efficient full-site integration has proven difficult. Many of the products are half-site integration reactions where either IN inserts only one end of the viral donor substrate into a circular DNA target or into other donors. In this report, we have purified recombinant HIV-1 IN at pH 6.8 in the presence of $MgSO_4$ that performed full-site integration nearly as efficiently as HIV-1 PIC. The size of the viral DNA substrate was significantly increased to 4.1 kbp, thus allowing for the number of viral DNA ends and the concentrations of IN in the reaction mixtures to be decreased by a factor of ~10. In a typical reaction at 37°C, recombinant HIV-1 IN at 5 to 10 nM incorporated 30 to 40% of the input DNA donor into full-site integration products. The synthesis of full-site products continued up to ~2 h, comparable to incubation times used with HIV-1 PIC. Approximately 5% of the input donor was incorporated into the circular target producing half-site products with no significant quantities of other integration products produced. DNA sequence analysis of the viral DNA-target junctions derived from wild-type U3 and U5 coupled reactions showed an ~70% fidelity for the HIV-1 5-bp host site duplications. Recombinant HIV-1 IN successfully utilized a mutant U5 end containing additional nucleotide extensions for full-site integration demonstrating that IN worked properly under nonideal active substrate conditions. The fidelity of the 5-bp host site duplications was also high with these coupled mutant U5 and wild-type U3 donor ends. These studies suggest that recombinant HIV-1 IN is at least as capable as native IN in virus particles and approaching that observed with HIV-1 PIC for catalyzing full-site integration.**

Integration of the retrovirus linear DNA genome into host chromosomes is essential for replication of human immunodeficiency virus type 1 (HIV-1). Preintegration complexes (PIC) are formed in the cytoplasm after reverse transcription of the viral RNA. Integrase (IN) in the PIC catalyzes the excision of two nucleotides adjacent to the phylogenetically conserved CA dinucleotide from the 3′-OH blunt ends of the viral DNA (7). After nuclear transport of the PIC, the two viral DNA ends are inserted by IN into the host genome in a concerted fashion, here termed "full-site integration" (8, 25, 44). The unpaired dinucleotides at the 5′ ends of the inserted viral DNA are removed, and the single-stranded gaps are repaired by a host cell DNA repair pathway (35). This process results in a short duplication of cell DNA ranging in size from 4 to 6 bp depending on the retrovirus species.

Significant progress in our understanding of retrovirus full-site integration was initiated by isolating PIC from virus-infected cells (8). Purified HIV-1 and murine leukemia virus (MLV) PIC are capable of incorporating ~15 to 50% of their viral DNA into an exogenously supplied DNA target as full-site integration products after 45 to 90 min of incubation at 37°C (6, 11, 13, 21, 39, 48). One report using HIV-1 PIC showed an ~95% incorporation of viral DNA into a target substrate where incorporation was essentially over after 45 min of incubation at 37°C (21). Cellular factors like barrier-to-autointegration factor (BAF) appear to play a role in maintaining stable MLV and HIV-1 PIC structures, thus promoting full-site integration by preventing integration of the viral DNA into itself, termed "autointegration" (12, 33, 48, 55).

Simplified integration assays using model linear retrovirus DNA with terminal U5 and U3 long terminal repeat (LTR) sequences, purified recombinant IN, and a DNA target were also developed (9, 16, 32, 45). These studies established that IN was the only viral protein necessary for both 3′-OH processing and DNA strand transfer activities.

Further progress in reconstituting the HIV-1 full-site integration process was made using IN derived from nonionic detergent lysates of virus particles (10, 22, 23). The 3′-OH recessed ends containing attachment (att) site sequences from two different viral donors (480 bp) were integrated by IN in a concerted manner into a circular DNA target (bimolecular

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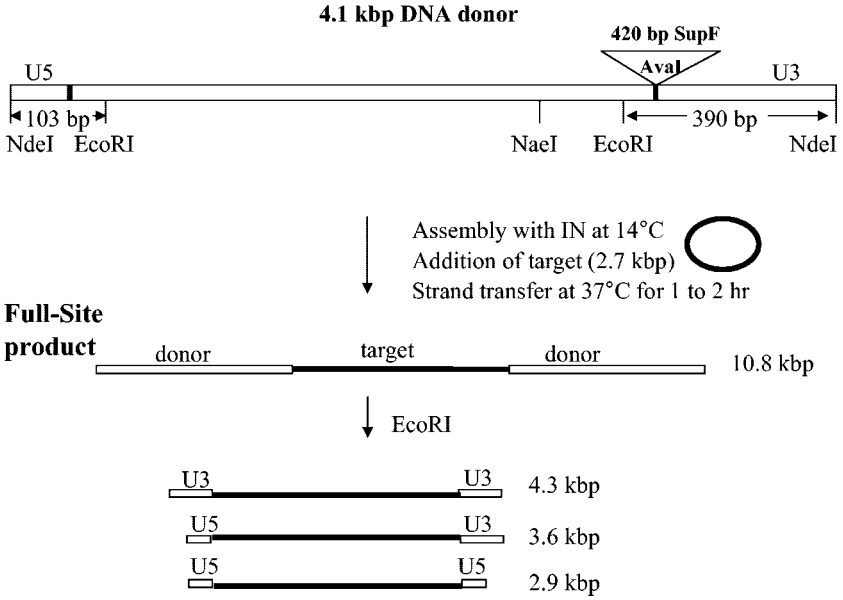


FIG. 1. Schematic representation of the 4.1-kbp linear DNA substrate, assembly conditions, and strand transfer analyses. The 4.1-kbp donor is depicted (top) with restriction sites and the 3′-OH recessed wt U5 and U3 termini. The donor contained a 420-bp DNA fragment (SupF) that was blunt-end ligated at the AvaI site. The dark line within the rectangular box identifies the approximate length of the LTR regions. IN was assembled with the donor substrate at 14°C followed by the addition of a circular target and strand transfer analysis at 37°C. Two donor ends are inserted by IN into the target producing a 10.8-kbp full-site product. Restriction digestion of the full-site products by EcoRI produced three restriction fragments depicted at the bottom.

reaction) (Fig. 1). In a typical reaction at 37°C for 30 min, 4 to 8% of the input donor DNA was incorporated into full-site products by these lysates. The fidelity of the HIV-1 5-bp host site duplications was ~70%. Subsequently improvements in purified recombinant HIV-1 (46) and simian immunodeficiency virus (24) IN provided an ~2-fold improved efficiency for producing full-site integration products over HIV-1 lysates. Recombinant HIV-1 IN using blunt-ended DNA donor substrates (~300 bp) with added HMGA1 is also capable of performing unimolecular full-site integration reactions where the two att ends from the same DNA donor are inserted into the DNA target (4, 29, 30). However, the primary products of the above integration reactions had one donor end either inserted into the DNA target (half-site integration) or into other donors.

In this study, recombinant HIV-1 IN purified at pH 6.8 in the presence of 10 mM $MgSO_4$ has the capacity to catalyze full-site integration with high efficiency and minimal synthesis of half-site integration products. A 4.1-kbp linear donor possessing 3′-OH recessed U5 (67 bp) and U3 (378 bp) LTR termini produced by NdeI digestion was employed. With this donor, we significantly lowered the number of DNA donor ends by a factor of ~10 as deduced from earlier studies (22, 23, 46). The concentrations of IN were also lowered proportionally, thus decreasing the frequency of unwanted half-site integration reactions. The full-site integration reaction efficiency was routinely in the 30 to 40% range with 5 to 10 nM IN for 120 min at 37°C. No cellular or viral factors were added. The fidelity of the 5-bp host site duplications was ~70% with coupled U5 and U3 donor ends. The rest of the sequenced clones contained repetitive small size bp deletions mirroring the same results obtained with HIV-1 lysates (22, 23). Other results suggest that IN must be kept in a sufficiently reduced state upon storage (−70°C) to maintain full-site integration activity and to avoid donor-donor integration reactions. In summary, recombinant HIV-1 IN under low protein concentrations without the requirement for organic solvents present possesses significant capacity for full-site integration.

## MATERIALS AND METHODS

**DNAs.** The 3.6-kbp linear retrovirus-like DNA substrate is a pBS+/− plasmid, formerly pBluescribe M13(+/−)(Stratagene), kindly provided by David Pauza. The plasmid harbors a 445-bp HIV-1 U5-U3 circle junction. A unique NdeI site was placed at the circle junction by site-directed mutagenesis (51). NdeI digestion produced a 5′-TA 2-base overhang at the terminus which is different than the normal 5′-AC overhang found on the wild-type (wt) HIV-1 3′-OH recessed ends. NdeI digestion linearized the modified plasmid that contained wt U5 (67 bp) and U3 (378 bp) att sequences, except for the 5′-TA overhang. For reference, this linear 4.1-kbp fragment is referred to as the "wt substrate." A DNA fragment containing the amber suppressor gene (SupF, ~420 bp) (46) was inserted adjacent to the U3 sequence to aid in the genetic selection of donor/target recombinants using MC1061/P3 cells (Invitrogen), thus producing a 4.1-kbp DNA substrate (Fig. 1). A 3.6-kbp mutant donor with two extra nucleotides beyond the conserved CA dinucleotide at the 3′-OH recessed end of U5 was also produced (see Fig. 7, row B). This donor lacked the SupF gene. Both donors were 5′-end labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (46). A third donor, termed "P2" (480 bp in length), containing terminal wt U3 and U5 att sites, was also isolated by NdeI digestion as previously described (46). The supercoiled target DNAs (2.7 kbp) used in the integration assays were plasmids pUC19 (with the EcoRI site destroyed) and pBSKΔ2-zeo (2.6 kbp), kindly provided by Karen Moreau (40).

**Purification of HIV-1 IN.** Expression and induction of HIV-1 IN (pNY clone) in bacteria and subsequent purification of IN were performed similar to an earlier report with minor modifications (46). The pH of the purification buffer (HEPES) was lowered to 6.8 with 10 mM $MgSO_4$ present. Fractions containing IN were divided into aliquots, frozen, and stored at −70°C. Aliquots of purified HIV-1 IN were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels which were stained with the Coomassie fluor

orange protein gel stain (Molecular Probes) (47) and analyzed by an Amersham Biosciences PhosphorImager. Concentrations of HIV-1 IN were determined using recombinant Rous sarcoma virus (RSV) IN as a standard (15).

**Integration assay.** Standard integration assay conditions were 20 mM HEPES (pH 7.0), 5 mM dithiothreitol, 10 mM $MgCl_2$, 25 μM $ZnCl_2$, 50 mM NaCl, and 10% polyethylene glycol. The amounts of donor and target DNA used were 10 ng and 50 ng per 20 μl of reaction volume, respectively. The target/donor molar ratio was 6. No organic solvents like dimethyl sulfoxide (DMSO) were used in the integration assays as previously described (46). Reaction volumes were varied from 100 μl to 200 μl to help control the concentration of IN in the reaction mixture. IN was preincubated with $^{32}$P-labeled DNA donor at 14°C for 15 min prior to the addition of supercoiled target DNA. The strand transfer reactions were carried out for 60 min to 120 min at 37°C. The reactions were stopped and products purified by phenol extraction followed by ethanol precipitation. The pellet was diluted in Tris-EDTA buffer. Samples containing equal number of DNA counts (Cerenkov) were loaded and subjected to electrophoresis on 1.5% gels for 13 or 15 h depending on the size of the donor DNA used in the assay. The gels were dried, and a PhosphorImager was used to analyze the labeled products.

**Analysis of full-site and half-site integration products.** The integration products produced with the wt 4.1-kbp donor containing the SupF (~420 bp) fragment were analyzed by EcoRI digestion. The full-site integration products were a mixture of U3-target-U3 (4.3 kbp), U5-target-U3 (3.6 kbp), and U5-target-U5 (2.9 kbp) (Fig. 1). Scaled-up reactions were performed to isolate sufficient quantities of the 3.6-kbp U5-target-U3 full-site products (Fig. 1). The isolated products were ligated and subsequently used to transform MC1061/P3 cells (Invitrogen) (41, 42). The donor-target junctions of individually isolated recombinants were sequenced using HIV-1 U3 and U5 sequence-specific primers (46). When the 3.6-kbp DNA donor containing the mutant U5 att site was used as substrate, the EcoRI-produced 3.1-kbp U5-target-U3 full-site integration products were isolated on agarose gels. The ligated U5-target-U3 full-site products were transformed into DH5α cells. The plasmids of the correct size were further identified by digestion of the unique NaeI site contained within the pBS+/− plasmid prior to DNA sequence analysis.

## RESULTS

A series of different purification protocols were performed on recombinant HIV-1 IN, including changing the pH (7.6 to 6.8) of the HEPES buffers with and without 10 mM $MgSO_4$. As previously reported (46), ~2 g wet weight bacterial pellets were used for purification of HIV-1 IN under low protein concentrations. The use of higher concentrations of crude bacterial lysates causes the precipitation of IN in solution, presumably due to the formation of insoluble IN-DNA complexes. The purity and concentrations of purified IN obtained in all of the tested procedures were similar. The results presented in this report were achieved from IN purified at pH 6.8 with 10 mM $MgSO_4$, which repeatedly produced the best preparations of IN for full-site integration. IN was purified to near homogeneity as previously reported (data not shown) (46).

**Full-site integration activity with a linear 4.1-kbp viral DNA donor and recombinant HIV-1 IN.** The linear 4.1-kbp donor substrate produced by NdeI digestion containing U5 (67 bp) and U3 (378 bp) sequences at its termini is shown in Fig. 1. We had previously reasoned (51) that lowering the number of viral DNA ends in the integration assay would allow lowering the concentration of IN, thus avoiding the production of unwanted strand transfer products. However, the specific activity of IN for full-site integration must also be high. HIV-1 IN was assembled with the 4.1-kbp donor for 15 min at 14°C prior to incubation at 37°C for strand transfer activity. By using the 4.1-kbp donor (10 ng), the optimum concentrations of IN for full-site integration activity were in the 5 to 15 nM range (Fig. 2A). At 5 nM IN, the molar ratio of IN dimers to donor ends is 14 to 1, respectively. Maximum synthesis of the 10.8-kbp full-site product (Fig. 1) was at approximately 10 nM IN in the assembly mixtures and subsequent reaction for 60 min at 37°C (Fig. 2A and Fig. 2B, lanes 2 to 4). Further increases in the concentration of IN allowed more synthesis of half-site over full-site integration products, although the total yield of integration products was essentially constant up to ~30 nM IN (Fig. 2A and 2B). Above 30 nM, inhibition of full-site integration activity occurred faster than that observed for half-site activity with increasing concentrations of IN.

We further investigated the time-dependent synthesis and yields of the 10.8-kbp full-site products with HIV-1 IN at 5 nM. Interestingly, the total yields of full-site products (~35%) after 2 h of incubation at 37°C essentially doubled the 1-h reaction amounts with little increase in the half-site products with time (Fig. 3A and 3B). Few other strand transfer products were produced with this extended time of incubation. These minor species were localized near the top of the gel (Fig. 3A). The total yields of the HIV-1 full-site products are comparable to those observed with HIV-1 PIC at 45 to 90 min of incubation at 37°C (6, 11–13, 20, 21, 39). Similar results were also observed with avian myeloblastosis virus (AMV) and recombinant Rous sarcoma virus (RSV) IN titration experiments using 3.6- to 4.6-kbp DNA donors, although the synthesis of full-site products with avian IN at 5 to 10 nM is significantly faster (~3- to 4-fold) and with slightly higher yields (15, 50, 51).

**Factors affecting full-site integration activity by HIV-1 IN.** We previously demonstrated that a smaller size donor termed "P2" (0.48 kbp) containing wt U5 and U3 termini (generated by NdeI digestion) produced ~6 to 8% full-site integration products using either nonionic detergent-lysed HIV-1 virions (10, 22, 23) or recombinant IN (10, 46). We directly compared the activity of recombinant HIV-1 IN purified at pH 6.8 using the 4.1-kbp donor (Fig. 2A and 2B) and the smaller size donor (22, 23, 46) (Fig. 2C) in assays performed simultaneously. The contents of the assay mixtures for both donors were nearly identical, except that the P2 reaction mixtures contained 12% DMSO with HEPES at pH 7.6 (46). The 0.48-kbp donor (15 ng) with HIV-1 IN essentially produced the same full-site and other integration products (Fig. 2C and 2D) as previously shown (22–24, 46). Maximum incorporation of the P2 donor into full-site products was observed in the 30 to 50 nM range with IN. The molar ratio of IN dimers to P2 donor ends at 50 nM IN is 11 to 1, respectively. Also evident in the P2 experiments (Fig. 2C) are the numerous donor/donor products as well as other integration products that are the result of single and multiple insertions of either the U5 or U3 donor ends into the DNA target (10, 22, 23, 46). DMSO is present in some but not all strand transfer reactions with recombinant HIV-1 IN (10, 18, 19, 23, 30, 34, 46, 49). The results suggest that the concentration of IN, the number of donor ends, and the assay solution conditions play critical roles in the preferential production of full-site integration products and the elimination of other unwanted strand transfer products.

We also reasoned that an assembly step was necessary to maximize the formation of synaptic complexes capable of full-site integration prior to initiation of strand transfer at 37°C. A series of assembly protocols using the wt 4.1-kbp donor with recombinant HIV-1 IN (5 nM) were set up either at 14°C or ice at various NaCl concentrations. Efficient assembly of synaptic complexes occurred at 14°C within 15 min (Fig. 4A). Lowering

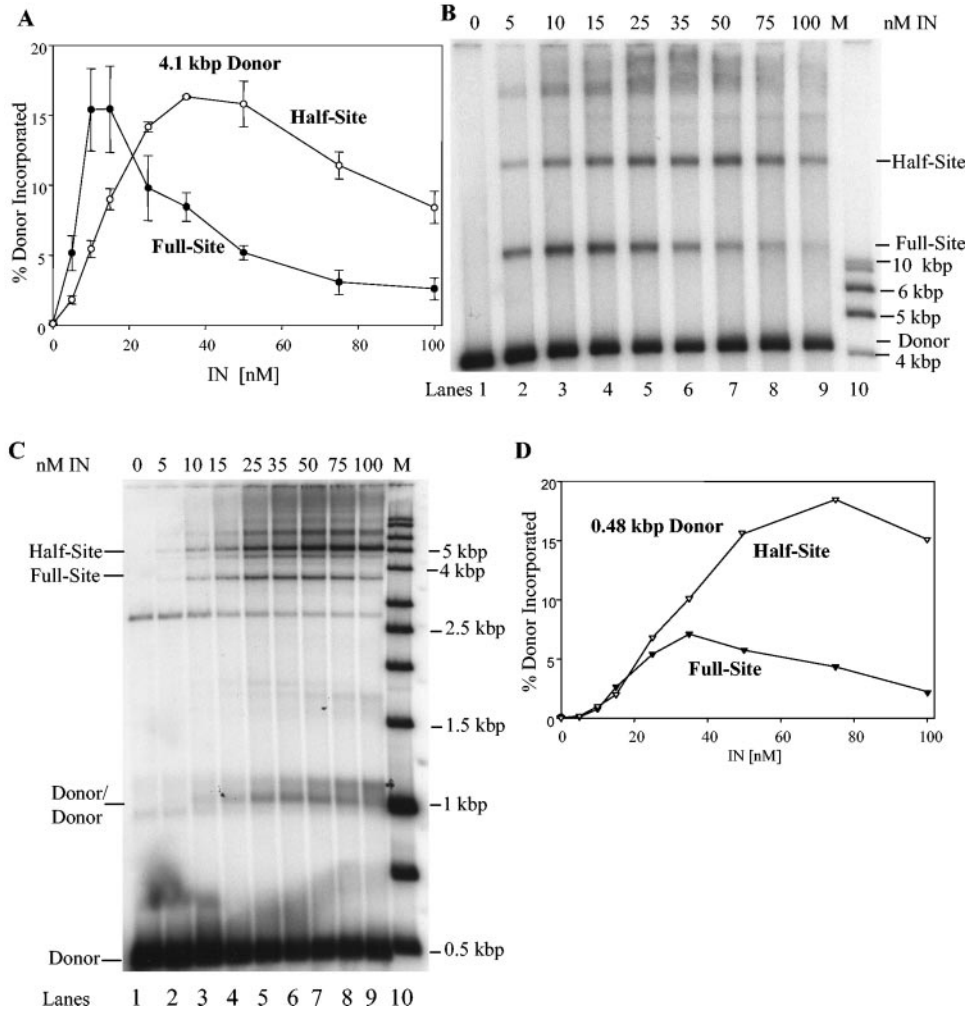


FIG. 2. Strand transfer activities of recombinant HIV-1 IN under different assay conditions. (A) IN was assembled at 0, 5, 10, 15, 25, 35, 50, 75, and 100 nM with either $^{32}$P-labeled 4.1-kbp or 0.48-kbp donors. The 4.1-kbp donor was assembled with IN for 15 min at 14°C prior to strand transfer for 60 min at 37°C. The 0.48-kbp donor (P2) was assembled with IN for 20 min on ice with DMSO prior to strand transfer for 20 min at 37°C (46). The samples were subjected to 1.5% agarose gel electrophoresis. The dried gels were analyzed by a PhosphorImager, and the percentage of donor incorporated into the integration products was determined from gels shown in panels B and C below. The full-site and half-site products using the 4.1-kbp donors (dark or open circles, respectively) were determined as marked on the figure. The presented data for the 4.1-kbp donor represent triplet measurements with error bar analysis. (B) The full-site and half-site products using the 4.1-kbp donor at various IN concentrations (top) as quantified in panel A. The products and the input donor are indicated on the right. The lane marked "M" on the right contains molecular weight markers as indicated on the right. (C) Same as panel B, except the 0.48-kbp donor was used. The donors/donor products are also shown on the left (46). A nonspecific DNA fragment was present in this P2 DNA preparation that is located in all lanes and migrates just above the 2.5-kbp marker. (D) The percentages of donor incorporated into half-site and full-site products were determined from the data in panel C with the 0.48-kbp donor.

the assembly temperature (incubation on ice) resulted in an approximate 35% decrease in the quantity of full-site products as compared to assembly at 14°C (Fig. 4A). Increasing the assembly times up to 1 h at 14°C with HIV-1 IN at 5 nM did not increase the quantities of full-site or half-site products using the 3.6-kbp donor (Fig. 4B) containing wt U3 and mutant U5 att ends (see Fig. 6 and Fig. 7, row B).

HIV-1 IN purification proceeded optimally using HEPES-NaOH buffer at pH 6.8. We had previously shown that IN purified at pH 7.5 exhibited maximal activity at pH 7.5 under standard assay conditions containing DMSO (46). The optimal pH for synthesis of the 10.8-kbp full-site integration product (Fig. 3) was 7.0 (data not shown). In addition, the concentration of NaCl in the assay mixture for full-site integration changed from an optimum of ~100 mM (46) to ~50 mM NaCl (Fig. 4A). These observations suggest that recombinant HIV-1 IN displays sensitivity to purification protocols as well as strand transfer assay conditions to produce full-site integration products.

Interestingly, the total amount of all strand transfer products produced by IN purified at pH 7.5 and with DMSO in the reaction mixtures was ~30% of the input 0.48-kbp donor substrate, with only ~8% of the donor being incorporated into full-site integration products (46) (Fig. 2C). HIV-1 IN purified at pH 6.8 and lacking DMSO in the assay incorporated ~ 40% of the input wt 4.1-kbp donor into full-site products (Fig. 3).

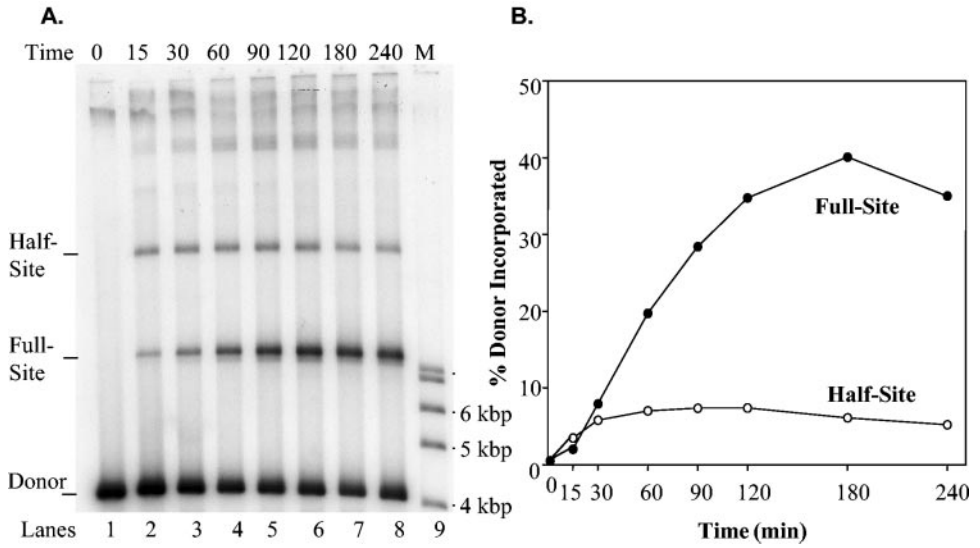


FIG. 3. Time-dependent synthesis of full-site and half-site integration products. (A) IN (5 nM) was preincubated with $^{32}$P-labeled 4.1-kbp DNA at 14°C for 15 min. Target DNA was added, and aliquots were removed and processed after 15, 30, 60, 90, 120, 180, and 240 min of incubation at 37°C (lanes 2 to 8, respectively). Lane 1 contained no IN. Lane 9, the 1-kbp DNA ladder markers with several identified on the right. The top two unmarked DNA fragments are 10 and 8 kbp (marked with dots), respectively (see Fig. 2, lane 10). (B) The graph depicts the amounts of full-site and half-site products produced in panel A.

These observations suggest that the specific activity of the recombinant HIV-1 IN was increased for full-site integration under these altered purification and strand transfer conditions. The optimum molar ratios of IN to donor DNA ends were similar in both of our studies for maximum synthesis of full-site integration products (Fig. 2A). However, the results in this report obtained with HIV-1 IN at 5 to 10 nM IN with the larger size donor (Fig. 3) demonstrated limiting the number of donor att sites in the reaction mixture is preferred.

Lastly, preparations of IN purified at pH 6.8 were the ones most efficient for catalyzing full-site integration. Several of these preparations did catalyze the formation of products that were identified as donor-donor products. These minor quantities of donor/donor products migrated just below the 10.8-kbp full-site products (Fig. 3; Fig. 5; and Fig. 6B, lane 3) and were evident in the absence of target in the reaction mixtures (data not shown). Preliminary studies suggest that the production of larger quantities of donor/donor products than those shown in this report was possibly related to aging of purified IN preparations over 2 to 3 months of storage at −70°C. Increasing the final concentration of dithiothreitol from 1 mM to 3 mM in these stored preparations maintained full-site integration activity better, thus decreasing the production of the donor/donor products. The results

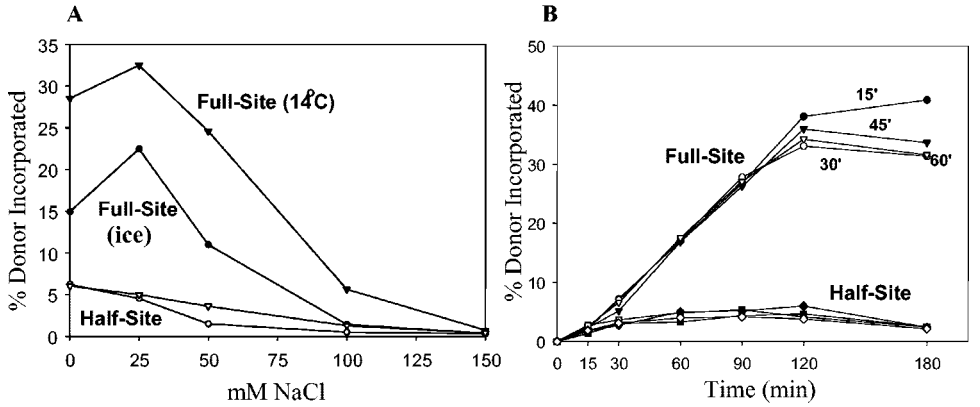


FIG. 4. Assembly properties of IN-DNA complexes capable of full-site integration. (A) IN (5 nM) was preincubated with the wt $^{32}$P-labeled 4.1-kbp DNA at 14°C or on ice for 15 min at various NaCl concentrations (bottom). Target DNA was added, and the samples were incubated at 37°C for 120 min. The quantities of integration products produced were determined and plotted against the NaCl concentrations. (B) The same assembly conditions at 14°C were used as described in panel A, except the times of assembly (●, 15 min; ○, 30 min; ▼, 45 min; ▽, 60 min) were different as indicated on the graph. The 3.6-kbp DNA donor was used. In simultaneously performed assays, aliquots were taken and assayed for strand transfer activities at 37°C as indicated at the bottom. The quantities of each product were determined and plotted. The top four lines are full-site products, while the bottom four lines are half-site products.

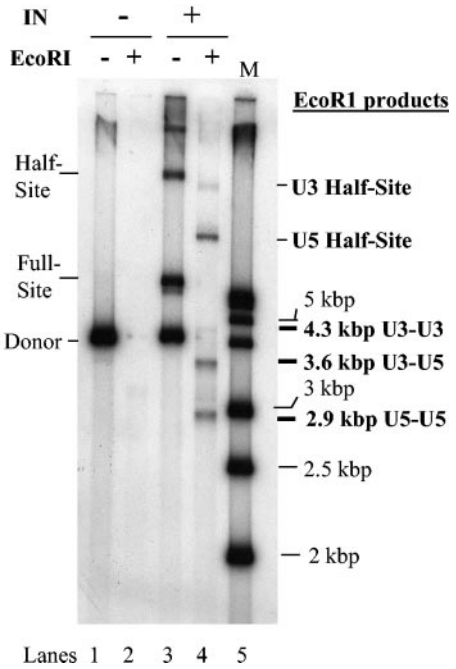


FIG. 5. Restriction enzyme analysis of strand transfer products produced by HIV-1 IN with the wt 4.1-kbp DNA donor. The strand transfer products were produced by IN at 5 nM (top). The integration products were divided into two nearly equal samples that were not (−) or were (+) digested by EcoRI. Lanes 1 and 2 were DNA donor without IN, while lanes 3 and 4 were with IN. Lane 5, the 1-kbp DNA ladder. The U3 and U5 half-site products (bold) and the 4.3-, 3.6-, and 2.9-kbp full-site products (bold) in lane 4 after EcoRI digestion are identified on the top right and bottom right, respectively. The positions of four DNA markers (not bold) are also illustrated on the right. The marker positioned between the 3.0- and 5.0-kbp markers is 4.0 kbp in length.

suggest that it was important to store recombinant HIV-1 IN in a higher reduced state.

**Restriction enzyme analysis and fidelity of host site duplications of the full-site integration products.** An accurate assessment of full-site integration products requires restriction enzyme analysis and sequencing of the donor-target junctions. The 10.8-kbp full-site products using the wt 4.1-kbp donor were synthesized and subjected to both analyses. EcoRI digestion of the 10.8-kbp donor removes both $^{32}$P-labeled ends from the molecule (Fig. 5, lanes 1 and 2) (Fig. 1). Restriction analysis of the 10.8-kbp full-site product (Fig. 5, lane 3) produced the anticipated 4.3-kbp (U3-target-U3) product that is barely visible and the 3.6-kbp (U3-target-U5) and the 2.9-kbp (U5-target-U5) DNA products (Fig. 5, lane 4). The higher activities of U5 att sites over U3 att sites by HIV-1 IN were previously demonstrated for both full-site and half-site integration products (4, 5, 7, 10, 23). Digestion of the half-site product (Fig. 5, lane 3) by EcoRI also produced the anticipated quantities of the U3 and U5 half-site products (Fig. 5, lane 4). Restriction analysis suggests that HIV-1 IN produced the correct full-site products.

Scale-up preparations of the 10.8-kbp full-site products were produced and subjected to EcoRI digestion. The 3.6-kbp products (U3-target-U5) were isolated from a wet gel (Fig. 5, lane 4), ligated, and transformed into MC1061/P3 cells. A total of 53 plasmids were sequenced. Approximately 70% of the recombinants possess the 5-bp host site duplications located at different sites in the DNA target (pBSKΔ2-zeo) (Table 1). In addition, 13 recombinants possess small size deletions that were similar to deletions produced by HIV-1 virion lysates (22) and recombinant HIV-1 IN (10, 22, 46). The results suggest that although HIV-1 IN produced higher levels of full-site integration products, the fidelity for host site duplications remained unchanged at ~70% (22, 26).

TABLE 1. Sequence analysis of donor-target junction sites produced by HIV-1 IN using wt 4.1-kbp donor[a]

| No. of: | | | | |
|---|---|---|---|---|
| Duplications | | Deletions[b] | | |
| 4 bp | 5 bp | 18 bp | 27–29 bp | 48–50 bp |
| 2 | 34 | 3 | 4 | 6 |

[a] The sequenced clones were derived from the U3-target-U5 DNA (3.6 kbp) isolated from agarose gels (Fig. 5, lane 4) and after transformation of the DNA into MC1061/P3 cells.
[b] Four deletions of 2, 82, 92, and 623 bp in length were also observed.

**Effects of modifying U5 att site sequences on the fidelity of the HIV-1 5-bp host site duplication.** The HIV-1 reverse transcriptase naturally elongates the 3′ ends of the U5 LTR by one or two nucleotides in vivo without apparent effects upon replication (39, 43). Also, one to three double-stranded nucleotide extensions at the 5′ blunt end of U5 only slightly modified the ability of recombinant HIV-1 IN to cleave the DNA internally at the phylogenetically conserved CA dinucleotide in vitro (49) or with AMV IN (53). We tested whether the purified recombinant HIV-1 IN at pH 6.8 had the ability to recognize a modified but active U5 att end containing nucleotide extensions (Fig. 7, row B). To accomplish full-site integration at the natural CA dinucleotide position (Fig. 7, underlined sequences on U5), IN must cleave the modified U5 end at the conserved CA dinucleotide prior to coupling with the U3 att end for full-site integration. The extended U5 att site would allow us to examine whether the recombinant IN work properly under nonideal active substrate conditions.

As anticipated, the synthesis of the full-site integration products by IN using the mutant U5 att site with U3 was somewhat delayed (Fig. 6A) relative to that of the wt 4.1-kbp donor (Fig. 3) at 37°C, presumably because IN must first cleave at the internal conserved CA dinucleotide on mutant U5 (Fig. 7, row C). The production of full-site integration products continues like that observed with the wt 4.1-kbp donor possessing NdeI 3′-OH recessed ends (Fig. 3), suggesting that HIV-1 IN may be associating and dissociating with both the U3 and mutant U5 att ends for extended times of incubation. In contrast to the U3 att site on the 4.1-kbp donor (Fig. 5, lane 4) (5, 10, 22, 23), the U3 att site on the 3.6-kbp donor containing the mutant U5 end is equally as active as or more active than the mutant U5 att site for both half-site and full-site integration activities (Fig. 6B, lane 4). The majority of the sequenced host-target junctions derived from the full-site products possess the correct host site duplications as well as the insertion of the mutant U5 end into the target site at the conserved CA dinucleotide (Fig. 7, rows C and D). In summary, the newly purified IN at pH 6.8

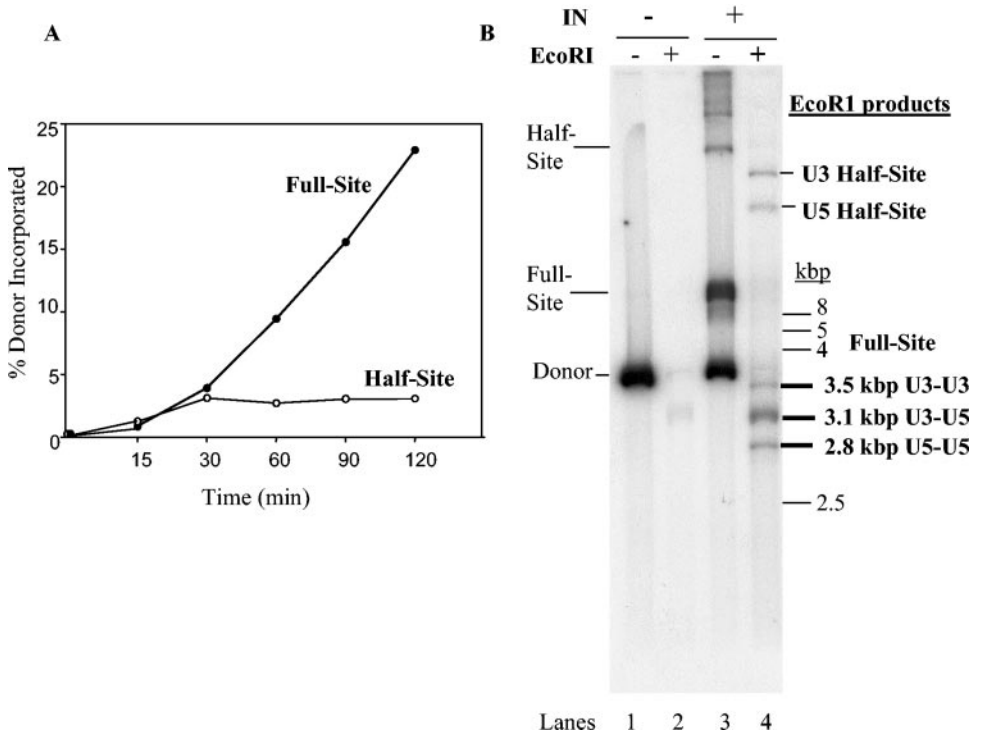


FIG. 6. Synthesis of full-site DNA products using a 3.6-kbp donor possessing wt U3 and mutant U5 att ends. (A) The modified U5 att end on the 3.6-kbp donor is described in Fig. 7, row B. Standard reaction conditions were used as described in the legend to Fig. 3 using IN at 5 nM. The graph depicts the amount of full-site and half-site products produced versus time of incubation at 37°C. (B) The U5 end of the 3.6-kbp DNA donor was modified as described in Materials and Methods and as shown in Fig. 7. HIV-1 IN (5 nM) was assembled with labeled donor DNA for 15 min at 14°C. Target DNA was added for strand transfer at 37°C, and the products were removed and processed after 120 min. The products were and were not subjected to EcoRI digestion (top). Lanes 1 and 2 were mutant DNA without IN, while lanes 3 and 4 were with IN. The half-site and full-site products (bold) in lane 4 produced by EcoRI digestion are identified on the top right and bottom right, respectively. The positions of the 1-kbp DNA ladder marker (not bold) are also illustrated on the right.

has significant full-site integration activity with a nonideal active U5 att substrate.

## DISCUSSION

The assembly of synaptic complexes between HIV-1 IN and model viral DNA substrates has had limited success that mimics the efficiency and fidelity of the HIV-1 PIC for full-site integration. In this report, recombinant HIV-1 IN purified at a lower pH (pH 6.8) and at low protein concentrations (200 to 300 μg/ml) has improved qualities for full-site integration. The properties of IN have also changed at this low pH in that DMSO is not required for efficient strand transfer activities. Lowering the number of att donor ends with a corresponding decrease in IN concentration (5 nM) significantly improves the efficiency of full-site integration but not the fidelity for the 5-bp host site duplications observed with HIV-1 virus lysates (10, 22, 23). Only two major products are produced—the full-site and half-site integration products—using supercoiled DNA as target. The results suggest recombinant HIV-1 IN has properties that appear similar to native HIV-1 IN in virus particles and possibly in PIC.

The synthesis of full-site and half-site integration products by recombinant HIV-1 IN appears to mimic the properties associated with AMV and recombinant RSV IN (15, 51, 52). The increased efficiency for full-site integration is highly dependent on the protein/DNA ratios in the assay mixtures (Fig. 2 and Fig. 3) with both IN systems. Increasing the concentration of HIV-1 IN favors the increased synthesis of half-site products over full-site products when compared to avian IN. The overall yields of products produced with both systems are similar, but the rate of synthesis for full-site products with HIV-1 IN is significantly slower (Fig. 3). The reason for this slower rate is not apparent. The incubation times for HIV-1 PIC are in the 45- to 90-min range for generally incorporating ~15 to 50% of the viral DNA into exogenously added target DNA to produce full-site integration products (6, 11, 13, 20, 21).

AMV (53–55) and recombinant RSV IN (1, 15, 28, 52) produce the correct avian 6-bp host site duplications with greater than 95% frequency upon full-site integration in vitro. The ability of recombinant HIV-1 IN to produce the 5-bp host site duplications with fidelity as high as that observed with HIV-1 PIC needs further improvement. The fidelity for producing the 5-bp duplications with HIV-1 virus lysates (10, 22, 23), recombinant HIV-1 IN (Table 1; Fig. 7) (46), and recombinant SIV IN (24) is ~70%. Recombinant HIV-1 IN in the presence of HMGAI and using a DNA substrate with U3 and U5 blunt ends to produce a unimolecular integration product also has a similar or slightly higher fidelity for the 5-bp host site duplications (4, 30). The two ends of one donor molecule

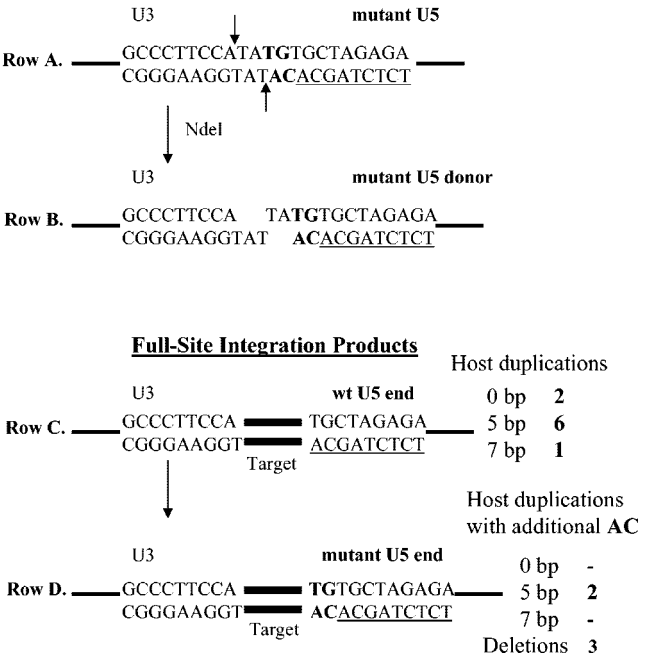


FIG. 7. HIV-1 mutant U5 att end with wt U3 produced host site duplications with a high fidelity. In row A, the circle junction for the wt U3 and mutant U5 ends in the 3.6-kbp plasmid DNA is shown. Two additional nucleotides (TG) were inserted into U5 and are shown in bold with the rest of the wt att site sequences underlined. The NdeI site at the circle junction was maintained. In row B, the plasmid DNA was digested with NdeI with the additional TG nucleotides (bold) shown. In row C, are shown the full-site integration products produced with wt U3 and mutant U5 ends that were isolated from agarose gels, followed by cloning and sequencing of the donor-target junctions. On the right in this row are shown the number of clones with 0, 5, and 7 bp of target site duplications in which the additional TG dinucleotide was removed prior to integration. In row D are shown clones that had the 5-bp target site sequence duplications but where IN did not remove the TG dinucleotides (bold) prior to producing the full-site products. There were three clones sequenced in this category that showed one each of the 18-, 27-, and 50-bp deletions.

(~300 bp) are inserted by HIV-1 IN into the target. Recently, the HMGA1 family proteins were shown not to be necessary for replication of HIV-1 or other retroviruses (3).

The ability of recombinant HIV-1 IN to form more stable synaptic complexes thus holding the two viral DNA ends correctly together may be enhanced by the addition of host factors like BAF found in both MLV (33, 48) and HIV-1 PIC (12, 36). BAF appears to stabilize PIC by preventing autointegration and thus stimulating full-site integration in vitro. A transcriptional activator, LEDGF, which interacts with HIV-1 IN (14, 17, 37, 38) and is a component of the HIV-1 PIC (37), appears to promote the trafficking of different lentiviral PIC into nuclei. Addition of recombinant LEDGF at 0.2 μM in reaction mixtures with recombinant HIV-1 IN at 0.6 μM significantly stimulated half-site strand transfer activities (14). Whether BAF and LEDGF under appropriate conditions enhance full-site integration activity of recombinant IN is unknown. Our current report suggests recombinant HIV-1 IN has improved qualities to catalyze full-site integration compared to those in previous reports but requires further investigation to fully match the properties associated with HIV-1 PIC.

AMV and recombinant RSV IN protect wt U3 and several gain-of-function att sites up to ~20 bp from the ends from DNase I digestion (15, 50, 51). This protection of att site sequences by IN at 14°C is correlated with full-site integration activity at 37°C. Interestingly, wt U5 sequences are not protected by avian IN up to ~20 bp from the ends but U5 is capable of full-site integration activity, suggesting that IN may recognize the U3 and U5 termini asymmetrically. We investigated whether recombinant HIV-1 IN was able to protect HIV-1 U5 and U3 att site sequences from DNase I digestion at 14°C under similar protein/DNA molar ratios achieved with avian IN. Single-ended wt U3 and U5 donors (3.6 and 3.4 kbp, respectively) labeled with $^{32}P$ at the 5′ att site were derived from the double-end-labeled 4.1-kbp donor (Fig. 1) by restriction digestion and isolation on agarose gels (51). HIV-1 IN does not protect wt U3 sequences up to ~20 bp when assembled with unlabeled wt U5 at 14°C (data not shown). However, the full-site integration activities of IN under these conditions with U3 were suboptimal to make any firm conclusions. Surprisingly, even though DNase I endonuclease activity is sensitive to position relative to the end of DNA up to ~10 bp (51), DNase I was unable to digest naked wt U5 up to approximately 20 nucleotides from the end. DNase I readily digested the U5 sequences beyond 20 bp from the end. The lack of DNase I digestion of the terminal U5 att site sequences on naked DNA (<20 bp) did not allow us to probe HIV-1 IN interactions with U5 with this technique. Other probing methods are necessary to investigate the interactions of HIV-1 IN with both U5 and U3 att sites under conditions that promote efficient full-site integration.

Recent findings have shown that inhibitors of HIV-1 IN in vitro effectively inhibit HIV-1 integration in cell culture as well as in an experimental animal model system (26, 27). Numerous earlier studies with inhibitors to HIV-1 IN as well as to HIV PIC in vitro provided insights into the problems and possibilities of selecting effective inhibitors to IN, thus preventing HIV-1/AIDS in humans (2, 31). Further development of reconstituted synaptic complexes capable of full-site integration in vitro using recombinant HIV-1 IN and model substrates may provide further biochemical insights into the mechanisms associated with IN inhibitors (26, 27).

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