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Juxtaposition of Two Viral DNA Ends in a Bimolecular Disintegration Reaction Mediated by Multimers of Human Immunodeficiency Virus Type 1 or Murine Leukemia Virus Integrase

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Integration of retroviral DNA involves a coordinated joining of the two ends of a viral DNA molecule into precisely spaced sites on target DNA. In this study, we designed an assay that requires two separate oligonucleotides to be brought together via interactions between integrase protomers to form a "crossbones" substrate that mimics the integration intermediate. The crossbones substrate contains two viral DNA ends, each joined to one strand of target DNA and separated by a defined length of target DNA. We showed that purified integrases of human immunodeficiency virus type 1 (HIV-1) and murine leukemia virus (MLV) could mediate a concerted strand cleavage-ligation between the two half-substrates at one or both viral DNA joining sites (*trans* disintegration). Another major product, termed fold-back, resulted from an intramolecular attack on the phosphodiester bond at the viral-target DNA junction by the 3′-OH group of the same DNA molecule (*cis* disintegration). The activity of integrase on the crossbones substrate depended on the presence of viral DNA sequences. For *trans* disintegration, the optimal length of target DNA between the viral DNA joining sites of the crossbones substrate corresponded to the spacing between the staggered joints formed on two opposite strands of target DNA during retroviral DNA integration in vivo. The activity of integrases on crossbones did not require complementary base pairing between the two half-substrates, indicating that the half-substrates were juxtaposed solely through protein-DNA interactions. The crossbones assay, therefore, measures the ability of integrase to juxtapose two viral DNA ends, an activity which heretofore has been difficult to detect by using purified integrase in conventional assays. Certain mutant integrases that were otherwise inactive with the crossbones substrate could complement one another, indicating that no single protomer in the integrase multimer requires a complete set of functional domains either for catalytic activity or for juxtaposition of the two viral DNA ends by the active multimer.

Integration of a double-stranded DNA copy of the retroviral genome into a chromosome of the host cell is essential for efficient viral replication (10, 27, 30, 32). Integration requires a virally encoded protein, integrase, and DNA sequences at the ends of the viral long terminal repeats (3, 21, 38). In vitro studies using oligonucleotides containing sequences of viral DNA ends showed that purified integrase of human immunodeficiency virus type 1 (HIV-1), murine leukemia virus (MLV), or Rous sarcoma virus can catalyze the two major steps of integration observed in vivo: (i) 3′-end processing, which involves the removal of two nucleotides from the 3′ end of each strand of linear viral DNA (4, 20, 25, 31), and (ii) 3′-end joining, a concerted cleavage-ligation reaction in which integrase makes a staggered, double-stranded break in the target DNA and ligates the recessed 3′ ends of the viral DNA to the 5′ ends of the target DNA at the cleavage site (4, 13, 19, 24). The chemical mechanism for both 3′-end processing and 3′-end joining is a one-step in-line transesterification (17). Integrase behaves as an enzyme (7, 22), and we have shown previously in vitro that the 3′-end joining step is reversible (8). With a substrate that corresponds to the 3′-end joining product, integrase correctly resolves the substrate into its viral and target DNA parts. The reverse reaction is called disintegration.

Mutational analyses of integrase have identified several distinct functional domains. Two of these are (i) the HHCC domain in the N terminus, named for its zinc finger-like motif $HX_{3-7}HX_{23-32}CX_2C$ (16, 23, 28, 37, 39), and (ii) the DD35E domain, located within a protease-resistant core, containing the motif $DX_{39-58}DX_{35}E$ (12, 16, 26, 28, 37, 41). Deletions or substitutions in the HHCC domain render the protein inactive in 3′-end processing and 3′-end joining reactions but still active in mediating disintegration, whereas mutations of the highly conserved aspartic or glutamic acid residues in the DD35E domain result in a loss of all catalytic activities (6, 12, 16, 26, 28, 37, 39, 41). A DD35E mutant protein, however, can complement an HHCC mutant and restore the 3′-end processing and 3′-end joining reactions (15, 35). Such complementation studies provide evidence that the active form of integrase is a multimer. A working model for the relationship between integrase, viral DNA, and target DNA at the 3′-end joining step is illustrated in Fig. 1. However, neither the organization and stoichiometry of the multimer nor the identities of the domains involved in multimerization are known. As depicted in Fig. 1, the integration reaction in vivo requires juxtaposition of the two ends of the viral DNA molecule and their coordinated integration into opposite strands of the target DNA at sites with a stereotypical spacing, which is a five-base 5′ stagger in the case of HIV-1 (29, 40). The requirements for juxtaposition of the two viral DNA ends and the multimerization that is presumably required to provide two functionally equivalent

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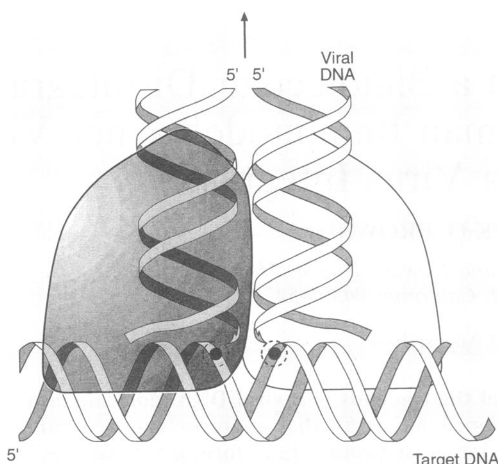

FIG. 1. Model for the configuration of integrase, viral DNA, and target DNA at the 3'-end joining step. The two ends of a linear viral DNA molecule are each bound by one or more integrase protomers. The two integrase protomers in this minimalist depiction are shown with a dyad symmetry (indicated by a vertical arrow). Each protomer has a single catalytic site (dashed circle). The half-arrows on the viral DNA denote the 3'-OH ends after 3'-end processing. During integration, two phosphodiester bonds (filled circles), one on each target DNA strand, are activated for nucleophilic attack by the free 3'-OH on the viral DNA end. The integration sites on the target DNA are staggered by 5 bp and are positioned across the major groove. The two viral DNA ends are, therefore, juxtaposed by integrase protomers in a stereotypical configuration relative to each other and the target DNA and approach the same face of the target double helix. Although not depicted here, it is equally possible that each viral DNA interacts with both integrase protomers (7) or with several integrase protomers, presumably in an assembly with a twofold axis of symmetry.

viral DNA binding sites in the enzyme complex have not been investigated.

Here we describe an assay that involves a novel DNA substrate that we termed crossbones. The assay requires that the two halves of the crossbones substrate be brought together via protein-DNA and, presumably, protein-protein interactions to form a structure that mimics the integration intermediate. We found that the integrases of HIV-1 and MLV can mediate a specific disintegration reaction with use of the crossbones substrate, indicating that integrase can functionally juxtapose two viral DNA ends and providing additional evidence that integrase functions as a multimer.

MATERIALS AND METHODS

Enzymes. Wild-type and amino-terminal mutants of HIV-1 integrase were expressed in *Escherichia coli*, using the T7 polymerase-promoter system (34), and purified as described previously (39). The D116N mutant of HIV-1 integrase was kindly provided by A. Engelman and R. Craigie at the National Institutes of Health. MLV integrase, expressed as a fusion peptide with glutathione S-transferase, was purified as described elsewhere (11). T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs; exonuclease-free Klenow fragment of *E. coli* DNA polymerase I was from United States Biochemical Corp.; *E. coli* exonuclease III was from Stratagene and New England Biolabs; terminal deoxyribonucleotidyltransferase was obtained from Boehringer Mannheim.

Nucleotides and oligonucleotides. Deoxyribonucleoside triphosphates were purchased from Pharmacia LKB. [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from ICN at a specific activity of 3,000 to 4,500 Ci/mmol. Oligonucleotides were purchased from Operon Technologies, Inc., and were purified by electrophoresis through a 15% denaturing polyacrylamide gel before use. To promote self-annealing and subsequent end labeling, purified oligonucleotides (100 pmol) were first heated in 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–80 μg of bovine serum albumin per ml for 3 min at 80°C and then slowly cooled to 4°C. The oligonucleotides were then labeled at the 3' end with exonuclease-free Klenow fragment of *E. coli* DNA polymerase I (10 U) and [α-^{32}P]dCTP in the presence of 160 pmol of unlabeled dCTP. The mixture was incubated at room temperature for 60 min, an additional 4 nmol of unlabeled dCTP was added, and the incubation was continued for another 60 min at room temperature. The final volume of the reaction was 50 μl. The reaction was stopped by heating at 75°C for 10 min. Unincorporated nucleotides were removed by centrifugation through a Sephadex G-15 column (0.9-ml bed volume). The labeled DNA was further purified by electrophoresis on a 15% polyacrylamide gel, extraction from the gel, and precipitation with ethanol. The labeled DNA was resuspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.1 M NaCl and then subjected to self-annealing by heating at 80°C for 3 min, then cooling slowly to room temperature, and finally chilling to 4°C.

Crossbones assay. The assay was designed to study the ability of integrase to mediate disintegration, a transesterification, between separate DNA molecules by virtue of the ability of the protein to form multimers. The reaction conditions for the crossbones assay were essentially the same as for disintegration (8). Typically in a 20-μl volume, the labeled DNA substrate (0.2 pmol) was incubated with HIV-1 integrase for 60 min at 37°C in a reaction buffer containing a final concentration of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 10 mM $MnCl_2$, 10 mM dithiothreitol, 0.1 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 0.05% Nonidet P-40. In reactions using MLV integrase, the reaction buffer contained a final concentration of 20 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 20 mM $MnCl_2$, 10 mM dithiothreitol, 10% glycerol, and 40 mM KCl. The concentrations of integrases used throughout this report refer to protomers and not necessarily active enzyme molecules. The reaction was stopped by adding 2.0 μl of 0.2 M EDTA (pH 8.0).

In reactions in which only the double-disintegrated product (DD product) and fold-back product of the crossbones assay were measured, a 2.5-μl aliquot of the reaction mixture obtained at the end of the 60-min incubation period was mixed with an equal volume of a solution containing 130 mM Tris-HCl (pH 8.0), 100 μg of bovine serum albumin per ml, 4 mM dithiothreitol, 32 mM $MgCl_2$, and 7.5 U of *E. coli* exonuclease III. The mixture was incubated at 37°C for 30 min, and the digestion was stopped by the addition of EDTA (pH 8.0) to 18 mM.

The reaction products were mixed with an equal volume of loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 90°C for 3 min before analysis by electrophoresis on 15% polyacrylamide gels with 7 M urea in Tris-borate-EDTA buffer. Quantitation of the products was carried out with a Molecular Dynamics PhosphorImager.

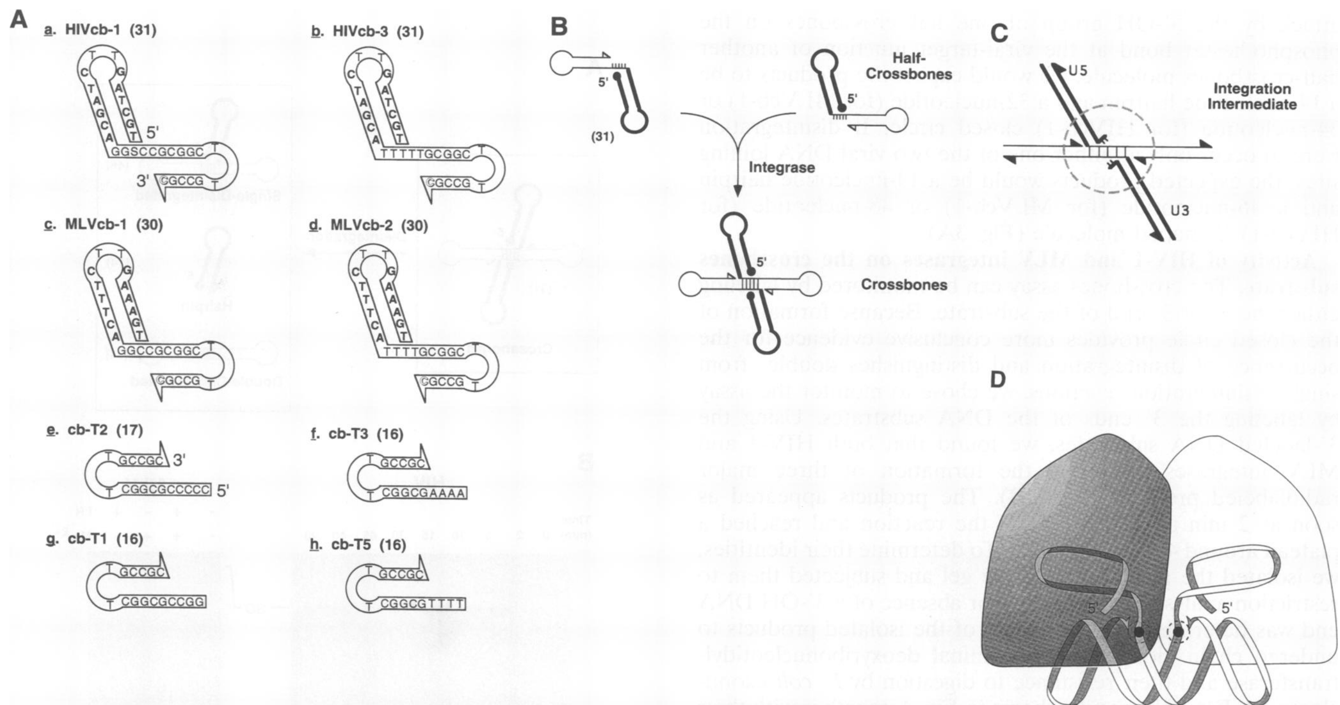

FIG. 2. DNA substrates and experimental design of the crossbones assay. (A) Sequences and predicted secondary structures of DNA substrates (half-crossbones) used in the crossbones assay. The crossbones substrate consists of two oligonucleotides (see below), and each half-crossbones is made up of a single oligonucleotide. The predicted secondary structure shown is based on the results of Blommers et al. (2). Boldface letters in the hairpin stem are DNA sequences of the HIV-1 (a and b) or MLV (c and d) U5 end. Plain letters are arbitrary DNA sequences representing target DNA. Shadowed, outlined letters at the 3′ DNA end (a to d) are [α-^{32}P]dCTP molecules that were filled in with exonuclease-free Klenow fragment of *E. coli* DNA polymerase I as described in Materials and Methods. S indicates that the nucleotide at that position is either C or G. Half-arrows denote the locations of the 3′-OH ends of DNA strands. The numbers in parentheses indicate the lengths in nucleotides of the oligonucleotides. Substrates a to d are termed half-crossbones. HIV-1 half-crossbones have five nucleotides in the single-stranded region, whereas MLV half-crossbones have four nucleotides. The two unpaired 5′ nucleotides of the viral DNA, which have no adverse effects on disintegration (8), are not included in the half-crossbones substrates. (B) Formation of the crossbones DNA substrate. Thick lines represent viral DNA, and thin lines represent target DNA. Short vertical lines represent unpaired nucleotides in the single-stranded region. A closed circle and a half-arrow denote the locations of the 5′-PO_4 and the 3′-OH ends of the strand, respectively. Through protein-DNA interactions with integrase, the half-crossbones can be brought together to form a crossbones structure that mimics the integration intermediate. The example shown is that of the HIV-1; a crossbones formed with MLV half-crossbones has 4 bp of target DNA between the two viral ends. (C) Diagram of the integration intermediate. Thick lines represent the U5 and U3 ends of the long terminal repeats of proviral DNA. Thin horizontal lines represent target DNA. Vertical lines represent the 5 bp (for HIV-1) of target DNA between the staggered breaks. The region of the intermediate mimicked by the crossbones substrate is highlighted by the dashed circle. (D) Juxtaposition of the two half substrates by integrase, drawn as in Fig. 1.

RESULTS

Experimental design. The prototypic DNA substrate (half-crossbones) is an oligonucleotide that folds to form two hairpin domains, corresponding to viral and target DNAs, respectively (Fig. 2). The secondary structure and the number of nucleotides in the hairpin loops shown in Fig. 2A were based on previous results obtained by using hairpins with similar DNA sequences (1, 2). Denaturing and native polyacrylamide gel electrophoresis of the half-crossbones showed that the DNA had a monomolecular folded structure and did not form bimolecular or higher-molecular-weight species (data not shown). The two hairpin domains are linked by a single-stranded region whose length equals the spacing between the coupled cleavage-ligation in the two strands of the target DNA during retroviral DNA integration in vivo. The spacing is characteristic for a particular retroviral integrase and is 5 and 4 bp, respectively, for HIV-1 and MLV (29, 38, 40). The viral sequences of the crossbones substrates HIVcb-1 and -3 (Fig. 2A, a and b) and MLVcb-1 and -2 (Fig. 2A, c and d) correspond to the DNA sequences of the U5 ends of the long terminal repeats of HIV-1 (HXB2) and MLV, respectively. The target DNA sequences are arbitrary and have no overt homology to the U5 or U3 region of the HIV-1 or MLV long terminal repeat. The assay requires that two separate oligonucleotides (half-crossbones) be brought together, presumably via interactions between integrase protomers, to form a crossbones substrate (Fig. 2B and D). The final structure of the crossbones is intended to mimic that of the integration intermediate (Fig. 2C; also see Fig. 1): it consists of two viral DNA ends with each end joined to one strand of the target DNA, at sites separated by 4 (for MLV) or 5 (for HIV) bp of target DNA. The juxtaposition of viral DNA ends required for the formation of the crossbones is, therefore, designed to resemble that required for concerted integration of two ends of a viral DNA molecule. Previously, using a Y-shaped oligonucleotide substrate corresponding to joining of a single viral DNA end to target DNA, we found that integrase can catalyze the reverse of the 3′-end joining step and correctly resolve the substrate into its viral and target DNA parts. We termed the reverse reaction disintegration (8). In the present study using the crossbones substrate, if correct disintegration were to occur at both viral DNA joining sites, mediated through a nucleophilic

attack by the 3′-OH group of one half-crossbones on the phosphodiester bond at the viral-target junction of another half-crossbones molecule, we would expect the products to be a 14-nucleotide hairpin and a 32-nucleotide (for MLVcb-1) or 34-nucleotide (for HIVcb-1) closed circle. If disintegration were to occur only at either one of the two viral DNA joining sites, the expected products would be a 14-nucleotide hairpin and a 46-nucleotide (for MLVcb-1) or 48-nucleotide (for HIVcb-1) Y-shaped molecule (Fig. 3A).

Activity of HIV-1 and MLV integrases on the crossbones substrate. The crossbones assay can be monitored by labeling either the 5′ or 3′ end of the substrate. Because formation of the closed circle provides more conclusive evidence for the occurrence of disintegration and distinguishes double- from single-disintegration reactions, we chose to monitor the assay by labeling the 3′ ends of the DNA substrates. Using the 3′-labeled DNA substrates, we found that both HIV-1 and MLV integrases promoted the formation of three major radiolabeled products (Fig. 3B). The products appeared as soon as 2 min after the start of the reaction and reached a plateau around 45 min (Fig. 3B). To determine their identities, we isolated the products from the gel and subjected them to restriction analysis. The presence or absence of a 3′-OH DNA end was determined by the ability of the isolated products to undergo chain elongation by terminal deoxyribonucleotidyltransferase and their resistance to digestion by *E. coli* exonuclease III. From the results shown in Fig. 4, together with their mobilities on a denaturing polyacrylamide gel (Fig. 3B), we identified the products as single-disintegrated (SD), DD, and fold-back. The SD product had a 3′-OH end (Fig. 4B) and resulted from a single disintegration reaction at one of the two viral-target DNA junctions. The DD product was a closed circle and was formed by disintegration at both viral-target DNA junctions (Fig. 4C). The fold-back product, like the DD product, was a closed circle since it was unable to undergo chain elongation and was resistant to exonuclease III digestion (Fig. 4D). Because of its relatively fast mobility on the gel and resistance to digestion by restriction enzymes *Eag*I and *Hae*III (Fig. 4D), we believe that the fold-back product was derived from disintegration of a single half-crossbones molecule (Fig. 5). The single-stranded region of the half-crossbones presumably formed a loop that positioned the 3′-OH end for an intramolecular nucleophilic attack at the viral-target DNA junction to form a circular molecule (7).

Although all three products are formed by transesterification, there is an important distinction in the source of the 3′-OH group involved relative to the phosphate group that undergoes transesterification. For the formation of SD and DD products, the attacking 3′-OH is provided in *trans* by a separate DNA molecule. In the fold-back, the nucleophile is located on the same DNA molecule as the transesterification site. We therefore use the terms *trans* disintegration for the former reaction and *cis* disintegration for the latter.

The results in Fig. 3 showed that both HIV-1 and MLV integrases acted on their respective crossbones substrates to produce the same family of products and that the two reactions followed a similar time course. However, the most abundant product for MLV integrase is the DD, whereas HIV-1 integrase forms two equally abundant products: the DD and the fold-back.

The optimal length of the overlap region of the crossbones matches the spacing between the staggered joints of viral and target DNA during integration in vivo. Integrase joins the two 3′ ends of viral DNA to staggered sites on the opposite strands of the target DNA. The spacing of the staggered sites at which the opposite strands are joined to viral DNA is constant for a

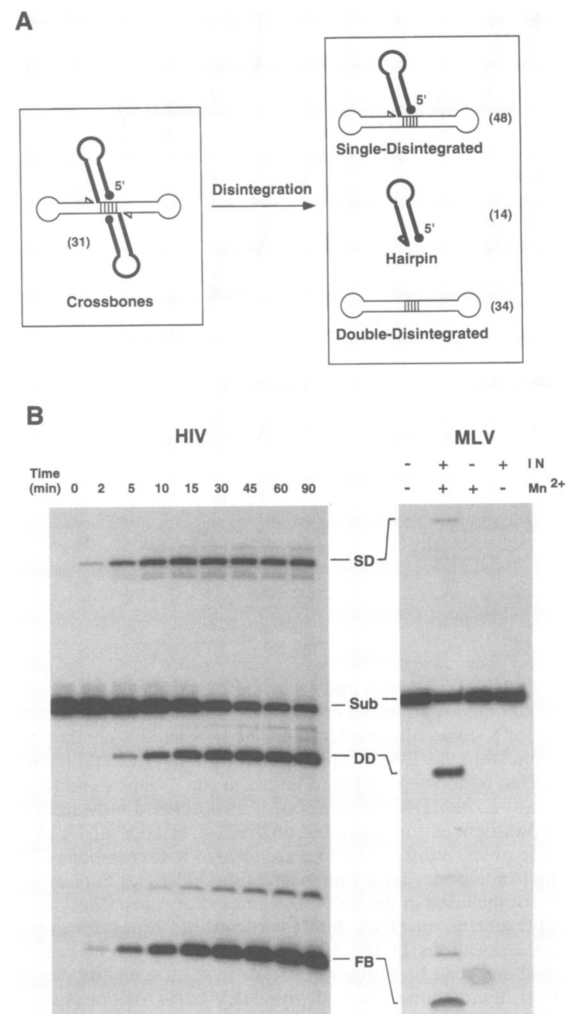

FIG. 3. Disintegration of the crossbones substrate by HIV-1 or MLV integrase. (A) Possible reaction products derived from the crossbones substrate. Correct strand cleavage and ligation of the crossbones substrate is expected to form a 14-nucleotide hairpin and a 48-nucleotide branched molecule if disintegration occurs at one of the two viral DNA joining sites (single-disintegrated). If disintegration occurs at both of the viral DNA joining sites, the same 14-nucleotide hairpin and a 34-nucleotide closed circular DNA will be formed (double-disintegrated). Symbols are the same as in Fig. 2B. The example shown is that of the HIV-1 reaction. The products expected from an MLV crossbones are a 14-nucleotide hairpin, a 46-nucleotide molecule, and a 32-nucleotide closed circular DNA. (B) Reaction products and time course of the crossbones assay. The half-crossbones was labeled at the 3′ end, and the reaction was carried out as described in Materials and Methods. In the left panel, 10 nM HIVcb-1 (Fig. 2A, a) was incubated with 100 nM HIV-1 integrase. At the indicated times after the start of the reaction, 10-μl aliquots of the reaction mixture were removed, and the reaction products were analyzed on a 15% denaturing polyacrylamide gel. In the right panel, 25 nM MLVcb-1 (Fig. 2A, c) was incubated with or without MLV integrase (IN; 100 nM) and Mn^{2+} as indicated at the top. Because of the location of the radioactive label, the formation of the 14-nucleotide hairpin was not directly monitored. Sub, substrate; FB, fold-back product. Unidentified bands shown on the gel (also see Fig. 7 and 8) are believed to represent products from reintegration of viral ends liberated by disintegration (8, 39).

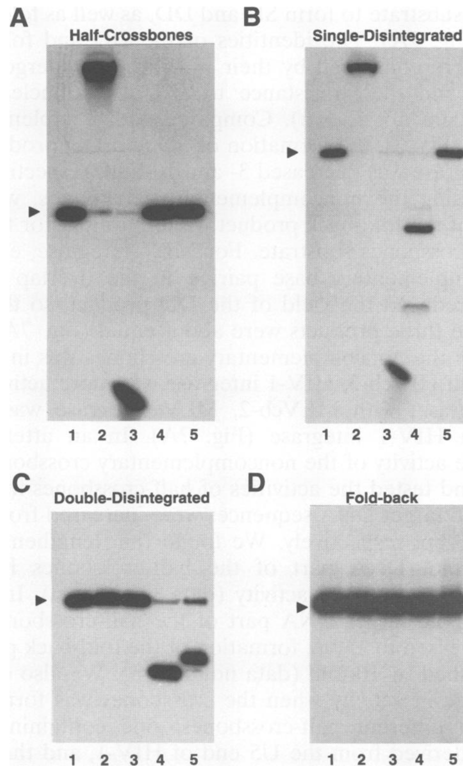

FIG. 4. Biochemical analyses of the reaction products. The major products and unreacted substrate of a reaction containing 0.1 μM MLVcb-1 (Fig. 2A, c) and 0.4 μM MLV integrase were isolated from a 15% denaturing polyacrylamide gel. The isolated substrate (A) and products (B to D) were subjected to various biochemical analyses: chain elongation with terminal deoxyribonucleotidyltransferase (lane 2), nuclease digestion with *E. coli* exonuclease III (lane 3), and restriction digestion using enzymes *Hae*III (lane 4) and *Eag*I (lane 5). Lane 1 in each panel represents the isolated substrate or product without further treatment. The products resulting from the above treatments were analyzed on a 15% denaturing polyacrylamide gel. Filled arrowheads indicate the position on the gel of the isolated substrate or product. The recognition sequence of *Hae*III is 5'-GGCC, and the enzyme is therefore expected to cleave the SD and DD products but not the half-crossbones substrate and the fold-back. The recognition sequence of *Eag*I is 5'-CGGCCG, and therefore the only molecule expected to be cleaved by the enzyme is the DD product.

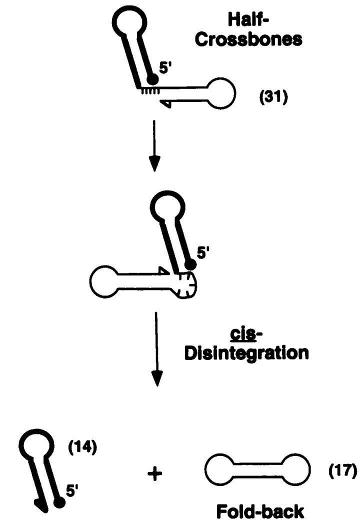

FIG. 5. Possible mechanism for formation of the fold-back product. Symbols are the same as in Fig. 2B. The numbers in parentheses indicate the lengths in nucleotides of the DNA molecules. The 3'-OH end of the half-crossbones substrate, either by virtue of the flexibility of the single-stranded region or through the action of integrase, may be in proximity to mediate cleavage-ligation at the viral-target DNA junction to form a 14-nucleotide hairpin and a 17-nucleotide closed circular fold-back product (7). We termed the reaction mechanism *cis* disintegration since the nucleophile involved is on the same DNA molecule as the transesterification site.

particular retrovirus. Since the structure of the crossbones substrate is designed to mimic that of the integration intermediate, we hypothesized that the activity of integrase on the crossbones substrate would be affected by the length of the complementary region between the two half-crossbones. The activities of different integrases on various DNA substrates showed an integrase-specific pattern: HIV-1 integrase was more active than MLV integrase when HIVcb-1 was the substrate; conversely, MLV integrase was more active than HIV-1 integrase with MLVcb-1 as the substrate (data not shown). The difference in activity, however, could have been due to a difference between the two crossbones substrates either in the sequence of the viral DNA component or in the length of the overlap region between the two viral DNA joining sites, which corresponds to the single-stranded region of the half-crossbones substrate. To test the effect of the length of the overlap region, we examined the activity of integrase on a series of half-crossbones substrates that had identical viral sequences (derived from the DNA end of HIV-1) and identical target sequences but different lengths of the single-stranded region (Fig. 6A). We found that a half-crossbones without a single-stranded region was completely inert (data not shown), whereas half-crossbones containing a single-stranded region ranging from three to seven nucleotides were used as substrates by HIV-1 integrase (Fig. 6B). The formation of SD and DD products, which were produced by *trans* disintegration and therefore presumably depended on the formation of a crossbones structure, was affected by the length of the single-stranded region (Fig. 6B). The highest activity was observed with a substrate containing a five-nucleotide single-stranded region (Fig. 6C), which corresponds to the spacing between the staggered joints of viral and target DNA generated by HIV-1 integrase (29, 40). The formation of fold-back, which did not require the formation of crossbones and was produced by *cis* disintegration, was comparable among the substrates containing three to seven nucleotides in the single-stranded region (Fig. 6C).

***trans* disintegration is mediated by multimerization of integrase, does not rely upon base pairing in the single-stranded region, and requires the presence of viral DNA sequence.** The DNA substrates used in previous experiments, HIVcb-1 (Fig. 2A, a), MLVcb-1 (Fig. 2A, c), and HIVcb-s3 to -s7 (Fig. 6A), contain a single-stranded region that is complementary to that of another identical DNA molecule. The SD and DD products could have been generated by *trans* disintegration between two half-crossbones that were brought together either by multimerization of integrase or by pairing of complementary bases in the single-stranded region. To rule out the latter possibility, we used substrates (HIVcb-3 and MLVcb-2; Fig. 2A, b and d, respectively) with noncomplementary bases in the single-stranded region. Despite the absence of complementary base pairing for juxtaposition of the two half-substrates, either integrase was able to act on its cognate noncomplementary

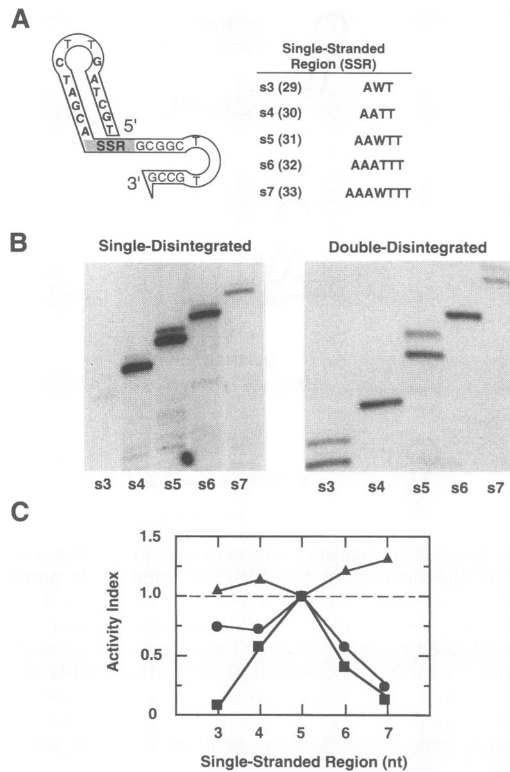

FIG. 6. Effect of the length of the single-stranded region on the crossbones assay. (A) DNA substrates. Except for the single-stranded (shaded) region, the viral and target DNA sequences were identical to those of HIVcb-1 (Fig. 2A, a). The length of the single-stranded region ranged from three to seven nucleotides (HIV-s3 to -s7). W indicates that the nucleotide at that position is either A or T. The terminal nucleotide of the half-crossbones was filled in with exonuclease-free Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dCTP. The specific activities of the various labeled substrates were similar (0.4 $\times$ 10^6 cpm/pmol). (B and C) Activity of HIV-1 integrase on half-crossbones substrates with various lengths of the single-stranded region. The reactions were carried out with 10 nM DNA substrates and 100 nM HIV-1 integrase under standard reaction conditions. After 60 min of incubation at 37°C, an aliquot of the reaction mixture was removed for analysis of the SD product. For the DD and fold-back products, an equal aliquot of the reaction mixture was treated with *E. coli* exonuclease III (see Materials and Methods) before analysis on a 15% denaturing polyacrylamide gel. The exonuclease treatment eliminated DNA molecules with ends and provided a better quantitation of the formation of the closed circular product. For half-crossbones containing odd numbers of nucleotides (nt) in the single-stranded region, the DD product appeared as a doublet on the denaturing gel. Because of the substrate design (see panel A), up to one-half of the DD product formed from the odd-numbered half-crossbones (HIVcb-s3, -s5, and -s7) could have a mismatched base pair in the complementary region. We believe that the additional band represents a different structural conformation adopted by the closed circle due to the presence of the mismatched base pair. Both bands in the doublet were quantitated for determining the formation of the DD product (see below). Under standard reaction conditions using HIVcb-s5 as the DNA substrate, values for formation of the labeled SD (■), DD (●), and fold-back (▲; not shown in panel B) products, when expressed as percentages of the total radioactivity, were 3.1, 2.1, and 61.8, respectively. The efficiency of *trans* disintegration of HIVcb-s5 was lower than that of HIVcb-1 (Fig. 3B) and is believed to be due to the difference in the GC content in the single-stranded region. The yield of each product with substrate HIVcb-s5 was arbitrarily normalized to 1.0 (denoted by the dash line). The yield of each product with other substrates, first calculated as a percentage of the total radioactivity for each substrate, was expressed as a relative activity, using the HIVcb-s5 reaction as the reference. A half-crossbones without any single-stranded region was inactive as a substrate (data not shown).

crossbones substrate to form SD and DD, as well as fold-back, products (Fig. 7A). The identities of the DD and fold-back products were confirmed by their inability to undergo chain elongation and their resistance to *E. coli* exonuclease III digestion (data not shown). Compared with complementary crossbones (Fig. 3), the formation of SD and DD products by HIV-1 integrase was decreased 3- and 10-fold, respectively, in reactions using the noncomplementary crossbones, whereas formation of the fold-back product was equivalent for the two classes of crossbones substrate. For MLV integrase, elimination of complementary base pairing in the overlap region drastically reduced the yield of the DD product, so that the yields of the three products were about equal (Fig. 7A). The activity with the noncomplementary crossbones was integrase specific: with HIVcb-3, HIV-1 integrase was more active than MLV integrase; with MLVcb-2, MLV integrase was more active than HIV-1 integrase (Fig. 7A). In an attempt to enhance the activity of the noncomplementary crossbones, we prepared and tested the activities of half-crossbones in which the viral and target DNA sequences were increased from 5 bp to 15 and 8 bp, respectively. We found that lengthening the viral or target DNA part of the half-crossbones had no stimulatory effect on the activity (data not shown). Interestingly, when the target DNA part of the half-crossbones was further increased to 15 bp, formation of the fold-back product was diminished by 10-fold (data not shown). We also did not see a change in activity when the crossbones was formed by mixing two different half-crossbones: one containing viral sequences derived from the U5 end of HIV-1, and the other containing viral sequences derived from the U3 end of HIV-1 (data not shown).

Both *trans* and *cis* disintegration were influenced by the viral DNA sequences. We found that substitution of the conserved CA/TG dinucleotide pair with TC/GA at the viral DNA end abolished the reaction mediated by HIV-1 integrase (Fig. 7A). To analyze the viral sequence requirement for a half-substrate to participate in the crossbones reaction, we carried out the reaction with MLV integrase and a 3′-end-labeled half-crossbones (Fig. 2A, d) in the presence of an unlabeled hairpin (Fig. 2A, f), which contains only target DNA sequences and a 5′ protruding end complementary to the single-stranded region of the half-crossbones (Fig. 7B). The labeled half-crossbones molecule, as expected, formed SD, DD, and fold-back products. The hairpin molecule, despite its potential to form complementary base pairs with the half-crossbones, did not compete and react with the half-crossbones substrate (Fig. 7B). We also did not detect any product formation when the identical reaction was repeated with an unlabeled half-crossbones and a 5′-end-labeled hairpin molecule (data not shown). The result indicates that the reaction requires that the two half-substrates be brought together via the interaction between integrase and the viral DNA sequence. Thus, the critical requirement is the presence of a viral DNA end in each of the two participating half-substrates, not the ability to form base pairs in the overlap region between the two half-substrates. However, increasing the length or the GC content of the overlap region between the half-crossbones and the hairpin molecule (Fig. 2A, c and g, for MLV; substrates not shown for HIV-1) allowed HIV-1 and MLV integrase to mediate *trans* disintegration between the two oligonucleotides (data not

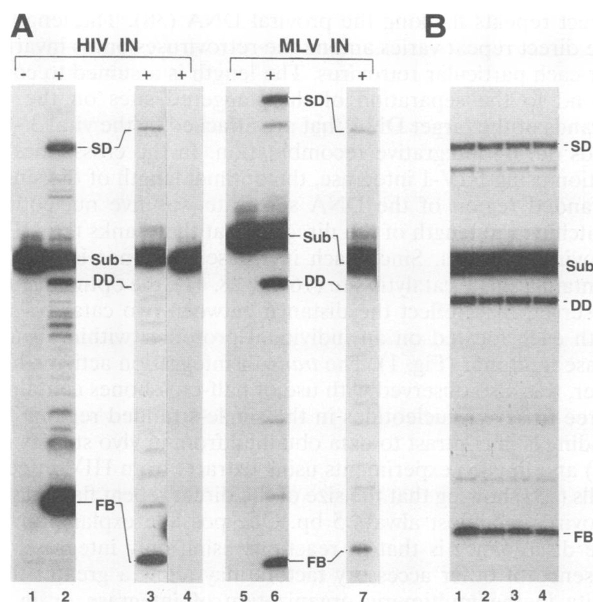

FIG. 7. (A) Formation of SD and DD products, using noncomplementary crossbones. Ten nanomolar HIVcb-3 (Fig. 2A, b; lanes 1, 2, and 7) and 25 nM MLVcb-2 (Fig. 2A, d; lanes 3, 5, and 6) were incubated with 100 nM HIV-1 (lanes 1 to 4) and MLV (lanes 5 to 7) integrase (IN), respectively, under standard conditions for the reaction analyzed. In lane 4, the half-crossbones substrate used had a DNA sequence identical to that of HIVcb-3 except that the CA/TG dinucleotide pair at the viral 3′ end was substituted with TC/GA. − and + represent absence and presence of integrase, respectively. (B) Presence of viral DNA sequence in the half-substrate is required for the crossbones reaction. Twenty-five nanomolar MLVcb-2 was mixed with 0 (lane 1), 12.5 (lane 2), 25 (lane 3), or 50 (lanes 4) nM cb-T3 (Fig. 2A, f) in the presence of 200 nM MLV integrase for 10 min at room temperature. $MnCl_2$ was then added to start the reaction, and the samples were incubated for 60 min at 37°C. The reaction between the hairpin and the half-crossbones, if it were to occur, would form a 32-nucleotide open loop. A synthetic oligonucleotide identical in length and similar in DNA sequence to the 32-nucleotide open loop showed a mobility between the input substrate (MLVcb-2) and the 46-nucleotide SD product on a 15% denaturing polyacrylamide gel (data not shown). Sub, substrate; FB, fold-back product.

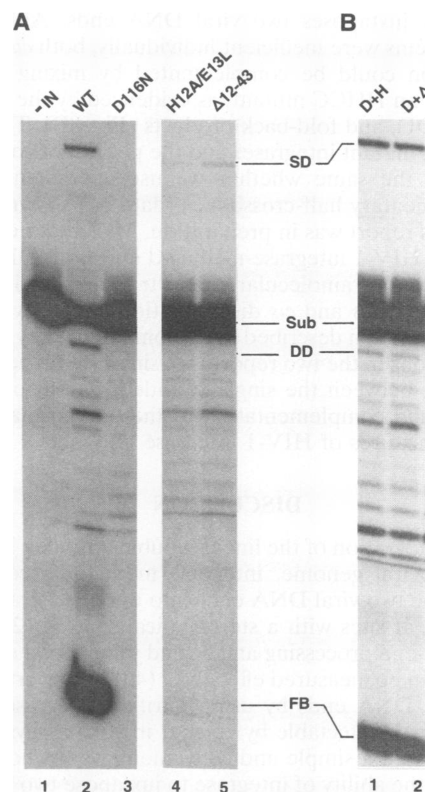

FIG. 8. Complementation of *cis* and *trans* disintegration by mutant HIV-1 integrases. (A) Activity of mutant HIV-1 integrases using the crossbones assay. Ten nanomolar HIVcb-3 (Fig. 2A, b) was incubated with 100 nM wild-type (WT; lane 2) or mutant (lanes 3 to 5) HIV-1 integrases under standard reaction conditions. The mutant integrases tested included two HHCC mutants (H12A/E13L [lane 3] and Δ12-43 [lane 4]) and a DD35E mutant (D116N [lane 5]). Δ12-43 contains a deletion of amino acid residues 12 to 43 at the N terminus. −IN, without integrase. (B) Complementation between different mutant integrases. The crossbones assay was carried out with HIVcb-3 as the DNA substrate. The final integrase concentration was 100 nM and was made up of two mutant integrases mixed at an equimolar ratio. Lane 1, a mixture of D116N and H12A/E13L (D+H); lane 2, a mixture of D116N and Δ12-43 (D+Δ). Sub, substrate; FB, fold-back product. The presence of the DD product was confirmed by exonuclease III treatment as described in Materials and Methods (data not shown).

shown). This is not unexpected since stable base pairing between the two oligonucleotides would result in a substrate resembling the standard Y-oligomer disintegration substrate (8). Neither HIV-1 nor MLV integrase could act on a mixture of half-crossbones and 5′-protruded hairpin that were not complementary in the single-stranded region, regardless of length or GC content (Fig. 2A, b and e; d and h) (data not shown).

Activity of mutant integrases and complementation between mutant integrases in the crossbones assay. Previous in vitro analyses of various mutants of HIV-1 integrase identified several distinct functional domains involved in the catalytic activities of the protein (12, 16, 26, 28, 37, 39, 41). Two of these domains were termed DD35E and HHCC. Amino acid substitutions in the DD35E domain, such as the D116N mutation, abolish all known catalytic activities of integrase: 3′-end processing, 3′-end joining, and disintegration. Variants containing substitutions or deletions in the HHCC domain are inactive in carrying out 3′-end processing and 3′-end joining reactions but can mediate disintegration. However, 3′-end processing and 3′-end joining activities can be restored by mixing a DD35E mutant with an HHCC mutant, suggesting that integrase forms functional multimers (15, 35). The nature of the multimer is not known, and the protein-protein interactions that allow complementation between mutant integrases may or may not be the same as those involved in juxtaposing two viral DNA ends. Since *trans* disintegration requires the integrase-mediated juxtaposition of two viral DNA sequences, and since it is unlikely that each integrase protomer has two binding sites with identical specificity for viral DNA, we believe that the crossbones substrate provides an assay for a distinct kind of multimerization between integrase protomers. When the activities of various mutant integrases were tested in the crossbones assay, we found that the D116N mutant was completely inactive in mediating either *cis* or *trans* disintegration; the HHCC mutants could not mediate *cis* disintegration but retained slight residual ability to mediate *trans* disintegration, since we could detect a trace amount of SD product (Fig. 8A). The HHCC domain is therefore not strictly required for the

process that juxtaposes two viral DNA ends. Although the mutant proteins were inefficient individually, both *cis* and *trans* disintegration could be complemented by mixing a D116N mutant with an HHCC mutant, as evidenced by the formation of the SD, DD, and fold-back products (Fig. 8B). The pattern of activity of mutant integrases and the pattern of complementation were the same whether we used complementary or noncomplementary half-crossbones (data not shown).

While this report was in preparation, Mazumder et al. (28a) reported an HIV-1 integrase-mediated intermolecular disintegration and an intramolecular strand transfer activity that are similar to the *trans* and *cis* disintegration, respectively, of the crossbones reaction described here. Some of the key properties of the reactions in the two reports are similar: independence of base pairing between the single-stranded regions of the half-substrates, and complementation of the bimolecular reaction by certain mixtures of HIV-1 integrase mutants.

DISCUSSION

During integration of the linear, double-stranded DNA copy of the retroviral genome, integrase mediates a coordinated joining of the two viral DNA ends into opposite strands of the target DNA at sites with a stereotypical spacing (29, 38, 40). Although 3′-end processing and 3′-end joining of a single viral DNA end can be measured effectively (42), a concerted joining of two viral DNA ends by using purified integrase is either inefficient or undetectable by current in vitro assays (5, 9, 18, 24). We devised a simple and convenient assay whose activity depends on the ability of integrase to juxtapose two viral DNA ends, an essential feature of retroviral DNA integration in vivo. We termed the novel DNA substrate half-crossbones and showed that integrase of HIV-1 or MLV is capable of placing two separate DNA molecules (half-crossbones) in juxtaposition. The expected dimeric structure formed is called a crossbones, and it mimics the structure of the integration intermediate. Transesterification of the crossbones by integrase involves a nucleophilic attack of the phosphodiester bond at the viral-target DNA junction of one DNA molecule by the 3′-OH end of another DNA molecule. Since the nucleophile and the transesterification site are present on separate DNA molecules, and the reaction leads to the release of viral DNA ends from a model integration intermediate; we called the reaction *trans* disintegration.

We believe that the *trans*-disintegration activities of HIV-1 and MLV integrases reflect their ability to form a multimeric structure that is able to bind and juxtapose two viral DNA ends. Our conclusion is supported by several properties of the *trans* disintegration. (i) Activity depends on the presence of viral DNA sequences on both half-substrates. Substitution of the conserved CA dinucleotide at the viral 3′ end or deletion of viral sequences abolishes the activity. (ii) *trans* disintegration does not require base pairing between the single-stranded regions of two half-crossbones. The two separate DNA molecules are, therefore, most likely placed in correct juxtaposition by an integrase multimer with two properly positioned binding sites for viral DNA. (iii) The optimal substrate for *trans* disintegration is integrase specific and depends on the length of the overlap region. The optimal length for activity corresponds to the spacing between the two staggered viral-target DNA joints made by integrase in vivo (see below). (iv) Certain mutations introduced into different domains of HIV-1 integrase render the protein inefficient or inactive in carrying out *trans* disintegration. The activity, however, could be restored by mixing complementary mutant proteins.

A hallmark of retroviral integration is the presence of short direct repeats flanking the proviral DNA (38). The length of the direct repeat varies among the retroviruses but is invariant for each particular retrovirus. The length is assumed to correspond to the separation of the staggered sites on the two strands of the target DNA that are attacked by the viral 3′-OH ends during integrative recombination. In the crossbones reaction using HIV-1 integrase, the optimal length of the single-stranded region of the DNA substrate was five nucleotides, matching the length of the direct repeat that flanks the HIV-1 provirus (29, 40). Since each integrase protomer is likely to contain a single catalytic site (16, 26, 28, 41), the optimal length observed may reflect the distance between two catalytic sites with each located on an individual protomer within an integrase multimer (Fig. 1). The *trans*-disintegration activity, however, was also observed with use of half-crossbones containing three to seven nucleotides in the single-stranded region. This finding is in contrast to data obtained from in vivo studies (29, 40) and in vitro experiments using extracts from HIV-infected cells (13) showing that the size of the direct repeat flanking the provirus is almost always 5 bp. One possible explanation for the discrepancy is that in reactions using only integrase, the absence of other accessory factors may allow a greater flexibility in the multimeric organization of integrase or in the structure of the DNA substrate. The increased flexibility may then be able to accommodate a larger range of length of the overlap region. The increased tolerance of variation in the length of the overlap region may also be simply attributed to the fact that substrate requirements for disintegration are generally less stringent than those for 3′-end processing and 3′-end joining reactions (7, 28).

In addition to *trans* disintegration, integrases of HIV-1 and MLV can also mediate transesterification using an unpaired half-crossbones molecule. We termed the reaction *cis* disintegration since the nucleophile and the transesterification site are both located on the same DNA molecule. The half-crossbones molecule can, therefore, undergo *cis* disintegration to form the fold-back product when it is unpaired or can undergo *trans* disintegration when it is paired with another half-crossbones molecule (Fig. 9). *trans* disintegration between two half-crossbones molecules at one or both viral-target DNA junctions leads to the formation of the SD or DD product, respectively. We have shown previously that integrases of HIV-1 and MLV can catalyze disintegration by using a substrate similar to the SD product (7). We do not know, however, whether the DD product is formed entirely in two distinct stages via the SD product or can be formed directly or processively from the crossbones substrate (Fig. 9). From the time course of product formation, we believe that at least some of the DD product is formed processively from the crossbones substrate.

Strand cleavage-ligation of the Y-oligomer and dumbbell substrate reported previously (7, 8) is basically analogous to *cis* disintegration in that both the nucleophile (3′-OH of DNA) and the transesterification site are on the same DNA molecule. In the Y-oligomer and dumbbell substrate, however, the 3′-OH group is placed in proximity to the phosphodiester bond by base pairing. During the formation of the fold-back product by *cis* disintegration of the half-crossbones, there is no base pairing to guide the nucleophile. The formation of fold-back, therefore, resembles that of cyclic dinucleotide formed during 3′-end processing of viral DNA: formation of the cyclic dinucleotide involves a direct nucleophilic attack by the hydroxyl group at the viral 3′ end on the phosphodiester bond two nucleotides upstream on the same DNA strand (17). Formation of the cyclic dinucleotide and fold-back suggests that the DNA substrate at the catalytic site undergoes struc-

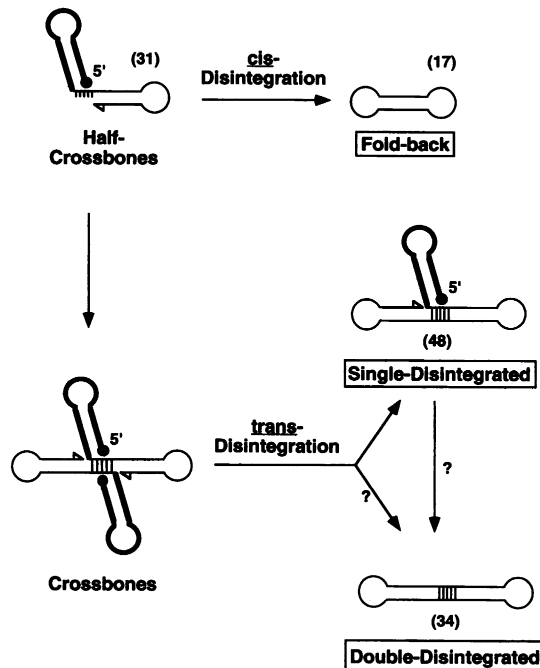

FIG. 9. Possible pathways of the crossbones reaction. Symbols are the same as in Fig. 2B. The unpaired half-crossbones substrate can undergo *cis* disintegration to form the fold-back product. When two half-crossbones, whether complementary or noncomplementary in the single-stranded region, are juxtaposed by multimerization of integrase, they form a crossbones structure. The crossbones can undergo *trans* disintegration to form SD and DD products. It is unclear (denoted by arrows with question marks) whether the DD product is formed directly from the crossbones or indirectly via the SD product. The 14-nucleotide hairpin, which corresponds to the released viral DNA end and can be formed by either *cis* or *trans* disintegration, is not shown.

tural distortion. Previous studies using the Y-oligomer and dumbbell substrate showed that integrase can tolerate distortion of the double helical structure of these substrates (7). From these results, we speculate that structural distortion of DNA substrates may be a general mechanism used by integrase during catalysis. Indeed, introduction of mismatched nucleotides at the viral DNA end stimulates 3'-end processing (33).

The crossbones reactions carried out by HIV-1 and MLV integrases are qualitatively identical. However, MLV integrase, in comparison with HIV-1 integrase, produces more DD product and less fold-back. Previous in vitro experiments using purified integrase and oligonucleotides showed that MLV integrase is more efficient than HIV-1 integrase in mediating concerted integration of two viral DNA ends (5, 9). The difference observed between the two integrases in the crossbones reaction may reflect a better ability of MLV integrase to form a multimeric complex in vitro with the DNA substrates.

Mutational analyses of HIV-1 integrase and complementation studies on mutant integrases suggest that HIV-1 integrase is active as a multimer and that different functions may be provided by discrete domains on different subunits (14, 15, 35). Integrase of Rous sarcoma virus has also been shown previously by Jones et al. (22) to form multimers. The organization of the multimer and the domains involved in multimerization are not known. Furthermore, integrase appears to be capable of forming more than one type of multimeric complex, such as a dimer, tetramer, and other higher-order forms (14, 15, 36). The activity and other properties of each multimeric complex and the relationships among the various types of complexes are presently not known. Our results showed that the crossbones reaction is useful in assaying the multimeric assembly that is responsible for juxtaposing two viral DNA ends. We found that the HHCC mutant, albeit at a very low level, could mediate *trans* disintegration, which indicates that the HHCC domain is not necessary for juxtaposition of the viral DNA ends. Further experiments using different combinations of wild-type and modified integrases and crossbones substrates may provide information on the amino acid residues important for assembly of the multimeric complex and juxtaposition of viral DNA ends and on the organization of functional domains in this complex. Protein-protein interactions between integrase protomers appear to be important for all known catalytic activities of integrase. Studies of the organization of integrase multimers and domains involved in multimerization are essential to understanding the molecular mechanism of retroviral integration.

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REFERENCES

1. **Antao, V. P., S. Y. Lai, and I. Tinoco, Jr.** 1991. A thermodynamic study of unusually stable RNA and DNA hairpins. Nucleic Acids Res. **19:**5901–5905.
2. **Blommers, M. J. J., J. A. L. I. Walters, C. A. G. Haasnoot, J. M. A. Aelen, G. A. van der Marel, J. H. van Boom, and C. W. Hilbers.** 1989. Effects of base sequence on the loop folding in DNA hairpins. Biochemistry **28:**7491–7498.
3. **Brown, P. O.** 1990. Integration of retroviral DNA. Curr. Top. Microbiol. Immunol. **157:**19–48.
4. **Brown, P. O., B. Bowerman, H. E. Varmus, and J. M. Bishop.** 1989. Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. Proc. Natl. Acad. Sci. USA **86:**2525–2529.
5. **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.
6. **Bushman, F. D., A. Engelman, I. Palmer, P. Wingfield, and R. Craigie.** 1993. Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. Proc. Natl. Acad. Sci. USA **90:**3428–3432.
7. **Chow, S. A., and P. O. Brown.** 1994. Substrate features important for recognition and catalysis by human immunodeficiency virus type 1 integrase identified by using novel DNA substrates. J. Virol. **68:**3896–3907.
8. **Chow, S. A., K. A. Vincent, V. Ellison, and P. O. Brown.** 1992. Reversal of integration and DNA splicing mediated by integrase of human immunodeficiency virus. Science **255:**723–726.
9. **Craigie, R., T. Fujiwara, and F. Bushman.** 1990. The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration in vitro. Cell **62:**829–837.
10. **Donehower, L. A., and H. E. Varmus.** 1984. A mutant murine leukemia virus with a single missense codon in *pol* is defective in a function affecting integration. Proc. Natl. Acad. Sci. USA **81:**6461–6465.
11. **Dotan, I., B. P. Scottoline, T. S. Heuer, and P. O. Brown.** Characterization of recombinant murine leukemia virus integrase. Submitted for publication.
12. **Drelich, M., R. Wilhelm, and J. Mous.** 1992. Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein *in vitro*. Virology **188:**459–468.

13. **Ellison, V., H. Abrams, T. Roe, J. Lifson, and P. O. Brown.** 1990. Human immunodeficiency virus integration in a cell-free system. J. Virol. **64**:2711–2715.
14. **Ellison, V., and P. O. Brown.** Unpublished data.
15. **Engelman, A., F. D. Bushman, and R. Craigie.** 1993. Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. EMBO J. **12**:3269–3275.
16. **Engelman, A., and R. Craigie.** 1992. Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. J. Virol. **66**:6363–6369.
17. **Engelman, A., K. Mizuuchi, and R. Craigie.** 1991. HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. Cell **67**:1211–1221.
18. **Fitzgerald, M. L., A. C. Vora, W. G. Zeh, and D. P. Grandgenett.** 1992. Concerted integration of viral DNA termini by purified avian myeloblastosis virus integrase. J. Virol. **66**:6257–6263.
19. **Fujiwara, T., and R. Craigie.** 1989. Integration of mini-retroviral DNA: a cell-free reaction for biochemical analysis of retroviral integration. Proc. Natl. Acad. Sci. USA **86**:3065–3069.
20. **Fujiwara, T., and K. Mizuuchi.** 1988. Retroviral DNA integration: structure of an integration intermediate. Cell **54**:497–504.
21. **Grandgenett, D. P., and S. R. Mumm.** 1990. Unraveling retrovirus integration. Cell **60**:3–4.
22. **Jones, K. S., J. Coleman, G. W. Merkel, T. M. Laue, and A. M. Skalka.** 1992. Retroviral integrase functions as a multimer and can turn over catalytically. J. Biol. Chem. **267**:16037–16040.
23. **Jonsson, C. B., and M. Roth.** 1993. Role of the His-Cys finger of Moloney murine leukemia virus integrase protein in integration and disintegration. J. Virol. **67**:5562–5571.
24. **Katz, R. A., G. Merkel, J. Kulkosky, J. Leis, and A. M. Skalka.** 1990. The avian retroviral IN protein is both necessary and sufficient for integrative recombination in vitro. Cell **63**:87–95.
25. **Katzman, M., R. A. Katz, A. M. Skalka, and J. Leis.** 1989. The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the in vivo sites of integration. J. Virol. **63**:5319–5327.
26. **Kulkosky, J., K. S. Jones, R. A. Katz, J. P. G. Mack, and A. M. Skalka.** 1992. Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. Mol. Cell. Biol. **12**:2331–2338.
27. **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.
28. **Leavitt, A. D., L. Shiue, and H. E. Varmus.** 1993. Site-directed mutagenesis of HIV-1 integrase demonstrates differential effects on integrase functions in vitro. J. Biol. Chem. **268**:2113–2119.
28a. **Mazumder, A., A. Engelman, R. Craigie, M. Fesen, and Y. Pommier.** 1994. Intermolecular disintegration and intramolecular strand transfer activities of wild-type and mutant HIV-1 integrase. Nucleic Acids Res. **22**:1037–1043.
29. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benson, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
30. **Panganiban, A. T., and H. M. Temin.** 1984. The retrovirus pol gene encodes a product required for DNA integration: identification of a retrovirus int locus. Proc. Natl. Acad. Sci. USA **81**:7885–7889.
31. **Roth, M. J., P. L. Schwartzberg, and S. P. Goff.** 1989. Structure of the termini of DNA intermediates in the integration of retroviral DNA: dependence on IN function and terminal DNA sequence. Cell **58**:47–54.
32. **Schwartzberg, P., J. Colicelli, and S. P. Goff.** 1984. Construction and analysis of deletion mutations in the pol gene of Moloney murine leukemia virus: a new viral function required for productive infection. Cell **37**:1043–1052.
33. **Scottoline, B. P., and P. O. Brown.** Unpublished data.
34. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
35. **van Gent, D., C. Vink, A. A. M. Oude Groeneger, and R. H. A. Plasterk.** 1993. Complementation between HIV integrase proteins mutated in different domains. EMBO J. **12**:3261–3267.
36. **van Gent, D. C., Y. Elgersma, M. W. J. Bolk, C. Vink, and R. H. A. Plasterk.** 1991. DNA binding properties of the integrase proteins of human immunodeficiency viruses types 1 and 2. Nucleic Acids Res. **19**:3821–3827.
37. **van Gent, D. C., A. A. M. Oude Groeneger, and R. H. A. Plasterk.** 1992. Mutational analysis of the integrase protein of human immunodeficiency virus type 2. Proc. Natl. Acad. Sci. USA **89**: 9598–9602.
38. **Varmus, H. E., and P. O. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
39. **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.
40. **Vincent, K. A., H. D. York, M. Quiroga, and P. O. Brown.** 1990. Host sequences flanking the HIV provirus. Nucleic Acids Res. **18**:6045–6047.
41. **Vink, C., A. A. M. Oude Groeneger, and R. H. A. Plasterk.** 1993. Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type 1 integrase protein. Nucleic Acids Res. **21**:1419–1425.
42. **Vink, C., and R. H. A. Plasterk.** 1993. The human immunodeficiency virus integrase protein. Trends Genet. **9**:433–437.