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Enzymatic Capability of HIS-Tagged HIV-1 Integrase Using Oligonucleotide Disintegration Substrates

Key Words
Integration mechanism
Disintegration
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Abstract
Disintegration, wherein a half-site integration substrate is resolved into separate viral and host DNA components via DNA strand transfer, is one of three well-established in vitro activities of HIV-1 integrase. The role of disintegration in the HIV-1 replicative cycle, however, remains a mystery. In this report, we describe the expression in *Escherichia coli* and purification of HIV-1 integrase as a fusion protein containing a 6xHis tag at its amino terminus. Integrase resolved dumbbell and Y-substrates optimally at pH 6.8–7.2 in the presence of 2 m*M* $MnCl_2$. Substrate requirements for intramolecular disintegration included a 10 base pair viral U5 LTR arm and a CA dinucleotide located at the 3′ end of the LTR. Disintegration was not sensitive to changes in the host DNA portion of the substrate. A dumbbell substrate with a 5′ oligo-dA tail also underwent disintegration. The released LTR arm with an oligo-dA tail was utilized as a template primer by several DNA polymerases indicating that disintegration occurred via nucleophilic attack on the phosphodiester bond located immediately adjacent to the CA dinucleotide at the 3′ end of the LTR. Coupled disintegration-DNA polymerase reactions provided a highly efficient and sensitive means of detecting disintegration activity. Integrase also catalyzed an apparently concerted disintegration-5′-end joining reaction in which an LTR arm was transferred from one dumbbell substrate molecule to another.

Integration of the HIV-1 genome into the host DNA chromosome is an essential step in the HIV-1 replicative cycle [1, 5, 6, 17, 18, 27, 32, 34, 37, 43]. It is accomplished in discrete steps, beginning with the processing of the 3′ ends of the viral genome, at a conserved CA dinucleotide in the viral LTRs, followed by joining of the processed ends to host DNA [25, 35]. The mechanism of DNA strand transfer, sometimes referred to as 3′-end joining, involves a nucleophilic attack of adenylate hydroxyls on phosphodiester bonds located 5 base pairs apart on either strand of the host DNA. This concerted transesterification reaction produces an intermediate in which the viral genome is flanked by 5-nucleotide gaps and is linked to the host DNA by its 3′ ends. Gap repair and removal of adjoining mispaired nucleotides (the so-called 5′-joining reaction) are thought to be carried out by host cell proteins [25, 35].

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Recombinant HIV-1 integrase is capable of distinct enzymatic reactions in vitro that are consistent with its role in the integration of HIV-1 DNA into the host cell chromosome in vivo [40]. Among these are endonucleolytic processing, in which a GT dinucleotide is removed from the 3′ ends of HIV-1 DNA, and 3′-end joining, in which the processed ends are joined to a host DNA target [3, 4, 13–16, 20, 21, 26, 28–30, 33, 36, 38, 39, 41, 42]. Using synthetic oligonucleotides configured as Y-shaped molecules to mimic integration intermediates, integrase also catalyzes a reversal of the 3′-end joining reaction, termed disintegration [7–11, 23, 31, 38]. Unlike the processing and joining reactions, disintegration does not have a known counterpart in vivo, making its role in the HIV-1 replicative cycle unclear.

We have expressed and purified recombinant HIV-1 integrase with a 6xHis tag fused at its amino terminus. We further characterized the disintegration reaction with respect to its substrate, pH and divalent cation requirements. Using a novel disintegration substrate with a 5′-oligodeoxyadenylate tail we directly confirmed the mechanism of disintegration. We also describe a novel method for detecting disintegration activity and present evidence for the participation of integrase in a concerted disintegration and 5′-end joining reaction.

Methods

Construction of an HIV-1 Integrase Bacterial Expression Vector

The 6xHis affinity tag Qiagen expression vector pQE 30 was cleaved with BamHI/SalI and the large fragment purified by agarose gel electrophoresis. The sticky ends were made blunt using the Klenow fragment of *Escherichia coli* DNA polymerase I. The modified expression plasmid, pQE 30 Δ, was circularized by ligation with T4 DNA ligase and the ligation products were used to transform the JM109 strain of *E. coli*. Plasmids were purified from bacterial extracts by elution from Qiagen columns. The small HindIII fragment of pCMV IN, which contains the HIV-1 integrase gene [19], was cloned into the HindIII site of pQE 30 Δ and the ligation products were used to transform the Kan^r *E. coli* strain M15 (pRep4). Individual bacterial colonies were screened for expression of integrase by Western blot analysis using a rabbit antiserum directed toward the keyhole limpet hemocyanin-modified nanopeptide KAQDEHEKY, located 5 amino acids from the N-terminus of the integrase protein. Unless indicated otherwise, all DNA-modifying enzymes were obtained from Boehringer Mannheim or New England BioLabs and used according to the manufacturer's instructions.

Expression and Purification of 6xHis-Tagged HIV-1 Integrase

Bacterial colonies of Kan^r M15 (pRep4) containing the pQE 30 Δ IN expression plasmid were picked from an LB agarose plate containing ampicillin and kanamycin and inoculated into 5 ml LB broth containing the same antibiotics. Bacteria were grown overnight at 37°C in beveled flasks in a rotary shaker. The next day, 10 ml of the overnight culture were inoculated into 250 ml of LB (containing kanamycin and ampicillin) and the bacteria were grown for 3–4 h to mid-log phase (OD 600 = 0.8) at 37°C. IPTG (isopropyl-thio-β-*D*-galactoside; BRL Life Technology Inc.) was added to a concentration of 2 m*M* and the culture was incubated for an additional 3–4 h to induce integrase expression. Bacteria were harvested by centrifugation for 30 min at 10,000 rpm in a JA14 rotor using a J2-21 Beckman centrifuge. Bacterial pellets were frozen at –20°C until use.

HIV-1 integrase was purified from bacterial pellets under denaturing conditions as described by Qiagen. Frozen pellets were thawed at room temperature and suspended in a 10-ml solution of buffer A (0.1 *M* Na phosphate, 0.01 *M* Tris-HCl, 6 *M* guanidine hydrochloride, pH 8.0). Bacteria were stirred for 30 min and centrifuged at 4°C and 10,000 rpm in a JA20 rotor using a J2-21 Beckman centrifuge. The supernatant was recovered and mixed with 2 ml of a 50% suspension of Ni^{2+} nitrilotriacetate (NTA) resin (Qiagen), previously equilibrated in buffer A. The mixture was stirred for 1 h at room temperature in order to allow the 6xHis-tagged integrase to bind to the beads.

The beads were pelleted by centrifugation at 700 *g* for 5 min in a clinical centrifuge, the supernatant was discarded and the beads resuspended in 20 ml of buffer A and allowed to settle in a polyethylene column. The resin was then washed with 10 ml buffer B (0.1 *M* NaH_2PO_4 buffer pH 8.0 containing 8 *M* urea) followed by successive washes with the same buffer at pH 6.3 (buffer C), pH 5.9 (buffer D) and pH 4.5 (buffer E). Integrase eluted in the pH 4.5 wash in a volume of 3–6 ml. The purified sample of integrase was dialyzed overnight in buffer B and applied to a fresh 2.0-ml bed of the 50% NTA resin previously equilibrated in buffer B. The resin was washed successively with 5 ml of buffers B and C and the final purified fraction of HIV-1 integrase was eluted in 3.0 ml of buffer E.

Renaturation of purified integrase preparations was carried out by slow stepwise dialysis against decreasing concentrations of urea (BRL Life Technology Inc.), deionized using BioRad Dowex 50. Dialysis was performed against 50 m*M* Hepes (4-[2-hydroxyethyl]-1-piperazinediethanesulfonic acid)-HCl buffer, pH 7.5, containing 1 m*M* dithiothreitol (DTT), 1.0 *M* NaCl and either 4 or 2 *M* urea for 24 h or more each time and finally twice against 50 m*M* Hepes-HCl, pH 7.5, 1 m*M* DTT, 1.0 *M* NaCl, 10% glycerol and 2 m*M* CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Boehringer), without urea. Occasionally a small precipitate formed during dialysis. The renatured enzyme was stored in 0.2-ml aliquots at –70°C and remained active for up to a year.

Synthetic Oligonucleotides

The following synthetic oligonucleotides were used in this study:

AE156:	5′GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC3′
AE157:	5′GAAAGCGACCGCGCC3′
AE146:	5′GGACGCCATAGCCCCGGCGCGGTCGCTTTC3′
U52:	5′ACTGCTAGAGATTTTCCACAC3′
DISXL-1:	5′ACTGCTAGAGATATCTCTAGCAGGGGCAGCCCCGGCGCAGCGCC3′
DISXL-5:	5′ACTGCTATAGCAGGGGCAGCCCCGGCGCAGCGCC3′
DISXL-9:	5′ACAACTAGAGATATCTCTAGTTGGGGCAGCCCCGGCGCAGCGCC3′
DISXL-3:	5′ACTGCTAGAGATATCTCTAGCAGGGGCCAGGCCCCGGCCGCAGCGGCC3′
DISPOL:	5′AAAAATGCTAGAGATATCTCTAGCAGGGGCAGCCCCGGCGCAGCGCC3′
DISPOL2:	5′AAAAATGCTAGAGATATCTCTAGCA3′
DISPOL3:	5′AAAAATGCTAGAGATATCTCTAGCATTTTT3′

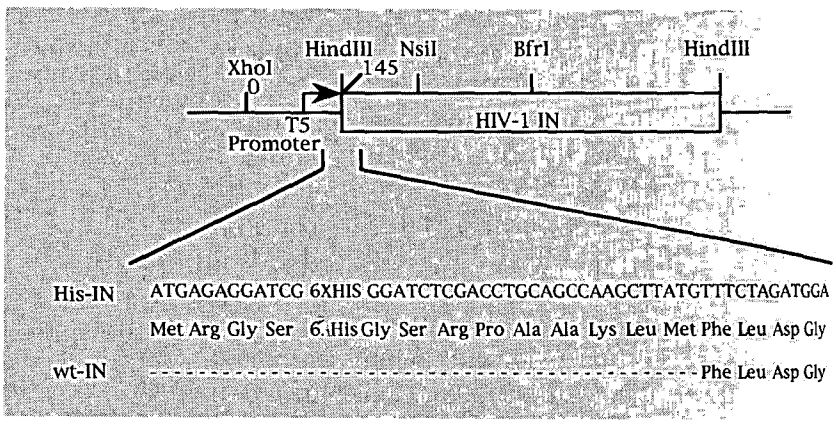


Fig. 1. Expression of HIV-1 IN from a pQE 30 Δ bacterial expression vector. The integrase gene was cloned as a HindIII fragment into the unique HindIII site wihin the MCS located at nucleotide 145 of the plasmid pQE 30 Δ. The coding region for integrase is depicted as a rectangular box. The solid line represents pQE 30 Δ. The NsiI and BfrI sites are unique within the integrase gene. The DNA sequence corresponding to the 6 histidines is CATCAC-CATCACCATCAC (not shown). In the 6xHis tag fusion protein, 19 extra amino acids are added to the amino-terminus of wild-type integrase. Except for the proximal methionine, the extra amino acids were encoded in the expression vector, as described in Results. The modified integrase is comprised of 307 amino acids and has a predicted molecular weight of 34,998.

Disintegration Reactions

Synthetic oligonucleotides were provided by Sheldon Biotechnology, Montreal, Que., Canada, and were partially purified by FPLC and further purified by electrophoresis in 20% nondenaturing polyacrylamide gels in 0.5 × TBE (45 m*M* Tris-borate, 1 m*M* EDTA, pH 8.0) at 350 V for 2 h. Prior to loading on the gels, oligonucleotides were boiled for 2 min and immediately cooled to 4°C. DNA was eluted from gel slices overnight or after 48 h at 37°C in 0.5 *M* ammonium acetate, 0.1% sodium dodecyl sulfate (SDS) and 10 *M* magnesium acetate. The DNA solution was extracted twice with an equal volume of *n*-butanol to remove the SDS and desalted on NAP-10 columns (Pharmacia).

Unless stated otherwise, disintegration reaction mixtures (25 μl) contained 50 m*M* Hepes-HCl, pH 7.0, 2 m*M* $MnCl_2$, 2 m*M* DTT, 10% glycerol, 40 m*M* NaCl, 0.2% CHAPS, 10 ng DNA, and 200 ng purified HIV-1 integrase. When DNA polymerase reactions were coupled to disintegration, reaction mixtures were supplemented with 1 vol 10 × pol buffer (100 m*M* Tris-HCl, pH 7.5, 50 m*M* $MgCl_2$, 10 m*M* DTT), 1 μ*M* TTP and 1 μCi α-^{32}P-TTP (3,000 Ci/mmol; ICN), and either 0.5 units Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase or exonuclease minus Klenow fragment (New England BioLabs). Reaction mixtures were incubated at 37°C for 1 h. Formamide dye mix (USB) was added (15 μl) and reaction mixtures were heated to 85°C for 5 min prior to analysis of the DNA products on denaturing polyacrylamide gels.

Typically, DNA was analyzed in 20% polyacrylamide (acrylamide:bisacrylamide 30:1) denaturing gels (0.5 mm thick, 20 cm long) containing 8 *M* urea in 100 m*M* Tris, 30 m*M* taurine, 0.5 m*M* EDTA. Electrophoresis was performed at 12 W, constant power, for 2 h. Following electrophoresis, gels were soaked in 10% methanol:5% acetic acid for 15 min followed by a final 5-min soak in either 50% methanol or water. Gels were dried under vacuum for 60 min at 50°C (methanol soak) or 80°C (water soak). Dried gels were exposed to Kodak Biomax X-ray film or XAR film (Kodak) for 16–48 h.

Radiolabeling Oligonucleotides with Polynucleotide Kinase

Polynucleotide kinase reactions were done in 50 μl and contained 1 μg purified oligonucleotide, 10 × kinase buffer (700 m*M* Tris-HCl, pH 7.6, 100 m*M* $MgCl_2$, 50 m*M* DTT), 125 μCi γ-^{32}P-ATP (3,000 Ci/mmol; Dupont-NEN) and 20 units polynucleotide kinase (Promega). Reaction mixtures were incubated at 37°C for 45 min. The enzyme was inactivated by heating at 80°C for 5 min. Radiolabeled DNA was separated from radioactive nucleotides by chromatography on NAP-10 columns in TE buffer (100 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 7.0).

Results

Purification and SDS-PAGE Analysis of HIS-Tagged Recombinant HIV-1 Integrase

The pQE 30 Δ IN bacterial expression vector used for these experiments produces a 34-kD HIV-1 integrase fusion protein in which HIV-1 integrase is fused at its amino-terminus to a 19 amino acid peptide with the sequence MRGSHHHHHHGSRPAAKLM, which includes a six histidine tag (fig. 1). The first 18 amino acids

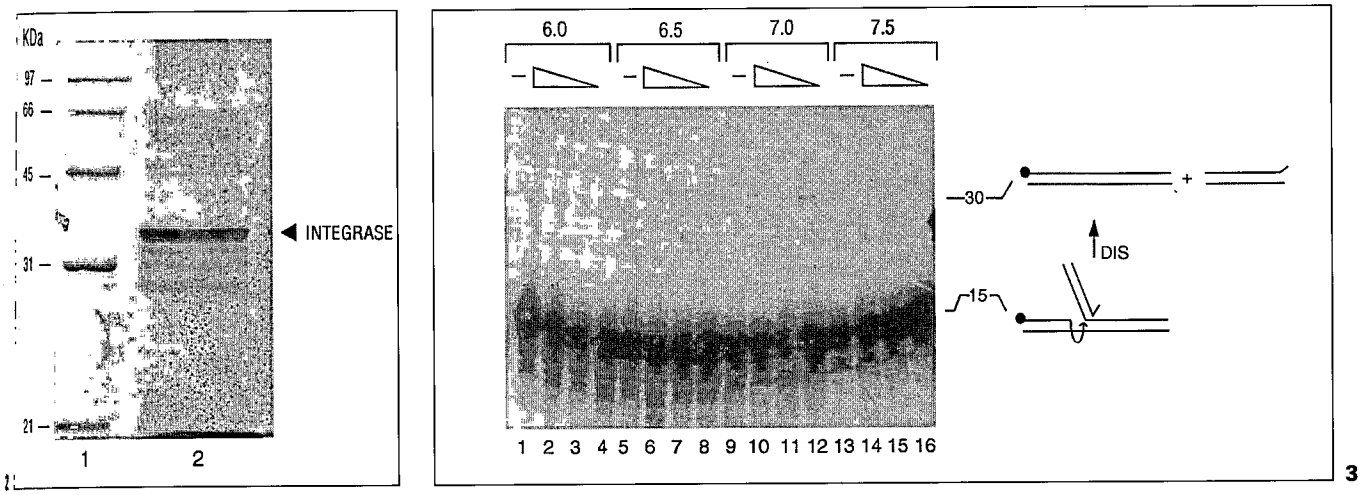


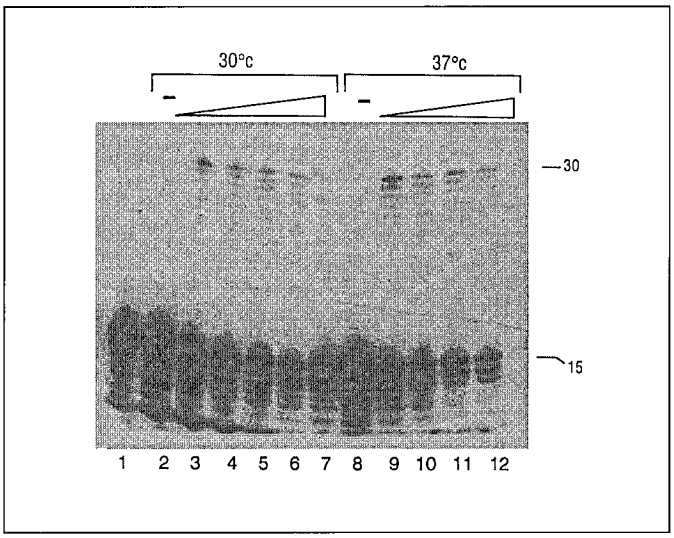
Fig. 2. Silver stain analysis of HIV-1 integrase purified by Ni^{2+}-NTA affinity absorption. Approximately 2 μg of purifed integrase (fraction E) was subjected to 12% SDS-PAGE electrophoresis and visualized by silver staining (BioRad). The molecular weights of protein standards (lane 1) are presented to the left of the integrase sample (lane 2).

Fig. 3. Schematic illustration of the disintegration reaction using a tetraoligomeric hybrid substrate and disintegration activity profile according to pH and $MnCl_2$ concentration. An illustration of the assay used to analyze the disintegration activity of integrase is presented to the right of the figure. Four oligonucleotides were hybridized together. Their nucleotide sequences are given in Materials and Methods. Oligomer AE157, a 15-mer, was ^{32}P-labeled at its 5′ end as indicated by the black dot. AE146 (a 30-mer) is represented by the solid line drawn below AE157. The oligomer AE156 (a 34-mer) is the bent line drawn as a partial hybrid (15 base pairs) with oligomer AE146. The U52 oligomer (a 21-mer) is hybridized to the upper portion (19 nucleotides) of AE156. The nucleotide sequence of this arm of the hybrid is homologous to the end of the U5 LTR in HIV-1 DNA. The unpaired region of U52 represents a 5′-terminal AC dinucleotide. Disintegration (separation of the U5 LTR arm from the rest of the hybrid molecule) is initated by nucleophilic attack of the 3′-OH of the radiolabeled 15-mer on the AE156 oligomer at the position indicated by the arrow. This reaction causes the AE157 oligomer (15-mer) to become covalently linked to a 15-mer segment of the AE156 molecule, thus producing a radiolabeled 30-mer. An autoradiogram depicting the results of disintegration assays done at different pH and $MnCl_2$ concentrations is presented in the left part of the figure. The numbers at the top of the autoradiogram are pH values. Reaction mixtures were constituted as described in Materials and Methods except that MES (morpholineethanesulfonic acid) buffer was used for reactions done at pH 6.0 and 6.5. Reactions done as controls without integrase are indicated by a horizontal line. The inclined planes represent decreasing concentrations of $MnCl_2$ fo 10, 5 and 2 m*M*. All reaction mixtures were incubated at 30°C for 1 h.

are derived from the pQE 30 Δ vector. The C-terminal methionine residue in the peptide was introduced into pQE 30 Δ along with the integrase gene which had a methionine residue engineered at its N-terminus [41]. The integrase fusion protein is readily detected in Western immunoblots of crude bacterial cell extracts using an antiserum directed toward a nonapeptide of integrase, namely KAQDEHEKY, which is located just 5 amino acids from the N-terminus [19]. The integrase fusion protein can be readily purified in a single step using nickel chelate chromatography. Such purified preparations of integrase contain a single major protein of 34 kD (fig. 2). Slightly shorter integrase-related proteins are also present. These truncated forms of integrase are missing differing amounts of carboxy-terminal amino acids as determined by Western blot analysis.

pH Optimum and Mn^{2+} Dependence of the Disintegration Reaction

The disintegration substrate used in our initial experiments was formed by hybridizing four oligonucleotides together, resulting in a hybrid consisting of an HIV-1 U5 LTR arm and a nonviral DNA segment (fig. 3). A 5′ ^{32}P-radiolabeled 15-mer is included in the hybrid in order to follow the disintegration reaction. The disintegration product expected with this substrate is a radiolabeled 30-mer which results when the U5 LTR arm is released from the substrate by the action of integrase. A radiolabeled 30-mer was produced when the reaction with integrase was performed at pH 7.0 in the presence of 2 m*M* $MnCl_2$ (fig. 3). The efficiency of this reaction was extremely low as only a very minor fraction of the starting material was recovered as a radiolabeled 30-mer. No radiolabeled 30-



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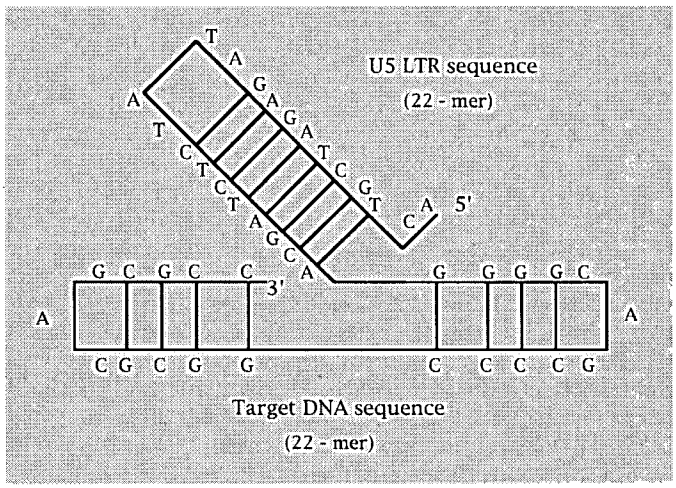

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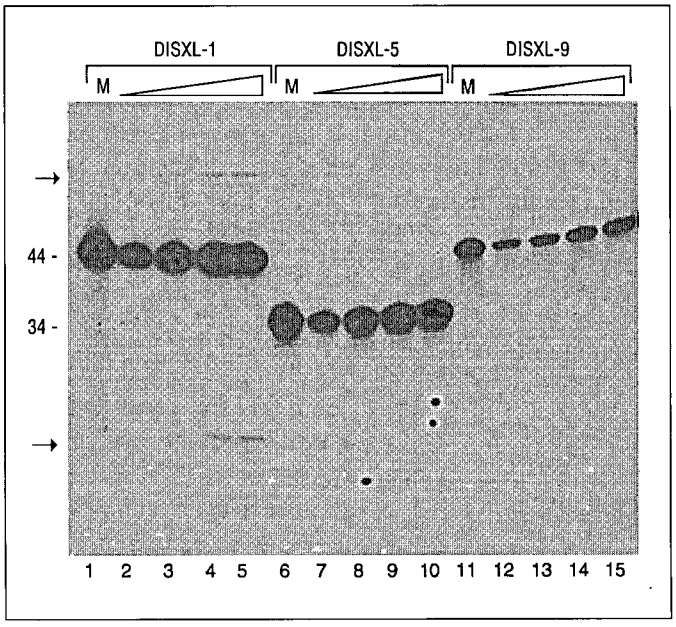

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mer products were seen when integrase was omitted from the reaction or when the $MnCl_2$ concentration was increased to 5 or 10 m*M* or when reactions were performed at pH 6.0, 6.5 or 7.5. Thus, the disintegration activity of integrase with this type of substrate displays relatively sharp optimum values with respect to pH and $MnCl_2$ concentration. The disintegration reaction proceeded equally well at 30 and 37 °C. Incubation of reaction mixtures for up to 6 h did not increase disintegration beyond that which occurred after 1 h of incubation (fig. 4).

A Dumbbell Substrate Can Undergo the Disintegration Reaction

We designed a 44-mer oligonucleotide dumbbell disintegration substrate DIS XL-1 for the next series of experiments. This oligonucleotide is predicted to fold into a 10 base pair duplex DNA arm representing the host DNA target of integration and a 10 base pair duplex U5 LTR arm with an unpaired AC dinucleotide at the 5′ end of the molecule (fig. 5).

Fig. 4. Time course of disintegration with a tetraoligomeric hybrid substrate. Requirements for optimal disintegration reaction conditions for the recombinant HIV-1 IN protein were examined at various times and at two different temperatures. Reaction mixtures were incubated at 30 or 37 °C for 0, 1, 2, 4 or 6 h. The inclined planes represent the increase in incubation times from left to right. Samples that did not contain integrase are indicated by a horizontal line at the top of the lane. The incubation temperature for each set of reactions is indicated at the top of the figure. The positions of the radiolabeled 15-mer oligonucleotides derived from the tetraoligomeric hybrid substrates and the radiolabeled 30-mer disintegration products are indicated at the right.

Fig. 5. Diagram of the DIS XL-1 dumbbell disintegration substrate. A 44-mer synthetic oligonucleotide is drawn in the base-paired configuration expected for the DNA sequence given. Features of the molecule include a 22-mer arm that matches the DNA sequence at the U5 end of the HIV-1 LTR. The 5′ end of the LTR arm terminates in an unpaired AC dinucleotide. A target DNA sequence composed of a GC-rich 22-mer segment is covalently continuous with the U5 LTR arm. Disintegration catalyzed by integrase releases the U5 LTR arm from the target DNA segment (see diagram in figure 7).

Fig. 6. Kinetics of the disintegration reaction with various dumbbell substrates differing in the U5 LTR. Disintegration reactions were conducted using 5′-end-labeled DNA substrates as described in Materials and Methods. Samples were incubated for 0, 15, 30 or 60 min as indicated by the inclined planes. Samples were then analyzed by electrophoresis in a denaturing polyacrylamide gel. The positions of the DNA substrates DIS XL-1 and DIS XL-9 (44-mers) and DIS XL-5 (34-mer) are indicated at the left according to their sizes. Unreacted DNA substrates were included in the lanes marked M. The arrows indicate the positions of the reaction products.

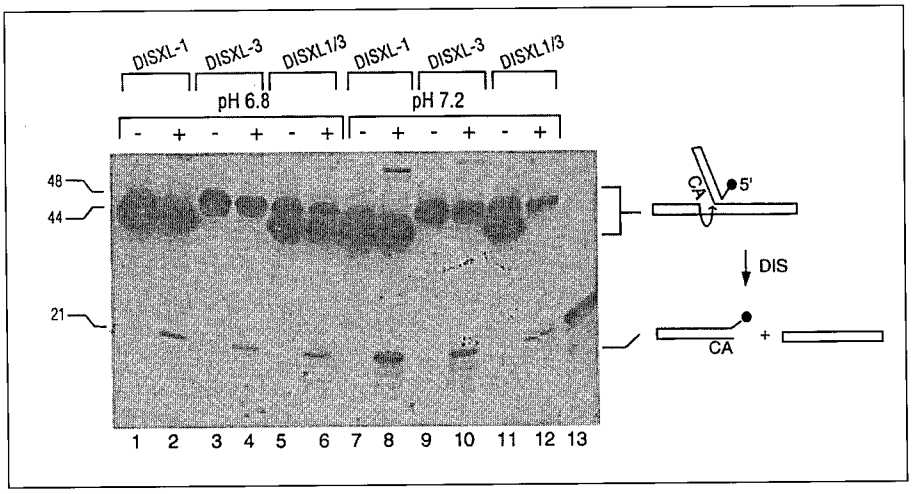

Fig. 7. Effects of the length of the target DNA segment and pH on the disintegration reaction. Disintegration reactions were compared with DIS XL-1 (44-mer), DIS XL-3 (48-mer) or mixed (DIS XL1/3) dumbbell substrates. DIS XL-3 has 2 extra GC base pairs in the target segment relative to DIS XL-1, each extra GC pair being located on either side of the LTR arm. The presumed mechanism of disintegration is illustrated at the right side of the figure. Molecules are radiolabeled at their 5′ ends with ^{32}P as indicated by the filled circles. The curved arrow indicates nucleophilic attack of a 3′-OH group on the phosphodiester bond adjacent to a CA dinucleotide in the U5 LTR. The disintegration products include the free LTR arm with a recessed 3′ end and a nonradioactive covalently closed circular molecule comprised of the host or target DNA segment. The positions of the starting substrates DIS XL-1 and DIS XL-3 are indicated at the left as 44 and 48, respectively. The position of the U5 LTR arm is designated as 21, which also reflects the position of a 21-mer oligonucleotide marker placed in lane 13. Reaction mixtures containing integrase are marked '+' and those without integrase are marked '–' at the top of each lane. Also indicated are the pH of each set of reactions and whether reaction mixtures contained DIS XL-1, DIS XL-3 or a mixture of these two substrates.

Incubation of 5′ ^{32}P-radiolabeled DIS XL-1 DNA with HIV-1 integrase led to the appearance of two radiolabeled products that were produced at the same rate over a 1-hour incubation period and in approximately equal amounts. One of these products comigrated with a 21-mer oligonucleotide marker while the other migrated much more slowly than the 44-mer substrate (fig. 6, 7). The structure of the larger radiolabeled reaction product and the mechanism by which it is produced are described below. The 21-mer likely represents the HIV-1 U5 LTR arm released by cleavage immediately adjacent to the 3′ side of the CA dinucleotide in the LTR portion of the 44-mer dumbbell substrate. As pointed out by others previously [7, 10, 11], a 22 nucleotide nonviral DNA circular molecule should also be produced in the disintegration reaction from DIS XL-1. Because the experiment was performed with a 5′ ^{32}P-radiolabeled substrate, the 22 nucleotide nonviral circular DNA molecule would not be radiolabeled since it is derived from the 3′ portion of the 44-mer DNA substrate. When a 3′-end-labeled DIS- XL-1 substrate was used in a comparable experiment, the 22 nucleotide circle was observed, as expected (data not shown). All further experiments described below were conducted with 5′-end-radiolabeled substrates.

Comparison of Different Dumbbell Substrates in the Disintegration Reaction

We next examined the effect of reducing the length of the U5 LTR arm in DIS XL-1 from 10 to 5 base pairs. When the shortened 34-mer substrate, DIS XL-5, was incubated with HIV-1 integrase, no disintegration products were observed (fig. 6). Similarly, no integrase reaction products were observed when the CA dinucleotide at the site of DNA strand transfer in DIS XL-1 was changed to TT in the DIS XL-9 substrate (fig. 6). We also tested another disintegration substrate, DIS XL-3, in which the host DNA portion was increased in length from 22 to 26 nucleotides. When incubated with DIS XL-3, HIV-1 integrase catalyzed the release of the 22 nucleotide U5 LTR arm as represented by a ^{32}P-radiolabeled DNA reaction product that comigrated with a 21-mer oligonucleotide marker (fig. 7; since the 22 nucleotide U5 LTR sequence is mostly a hairpin it is expected to run faster than a non-base-paired molecule of the same size, even under denaturing conditions). The analogous disintegration product

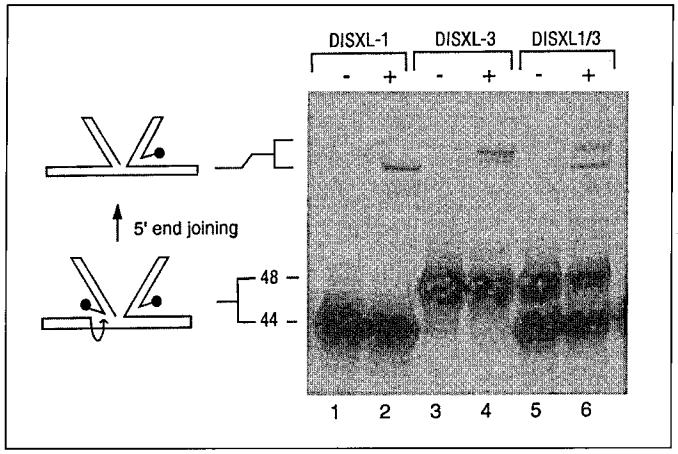

Fig. 8. Production of a high-molecular-weight DNA product by a presumptive concerted disintegration and 5′-end joining mechanism. Reactions were conducted as indicated in figure 7 and high-molecular-weight products were separated by prolonged electrophoresis in a denaturing polyacrylamide gel. The diagram at the left indicates a possible 5′-end joining mechanism for production of the high-molecular-weight DNA products. The curved arrow indicates nucleophilic attack of a 3′-OH group on a phosphodiester bond located adjacent to the 5′ unpaired dinucleotide in a free LTR arm. The dinucleotide is released and the remainder of the LTR arm becomes joined to the dumbbell substrate. The high-molecular-weight products therefore consist of a dumbbell molecule with two LTR arms, only one of which retains the 5′-terminal ^{32}P. The positions of the starting dumbbell substrates in the autoradiogram at the right are indicated by the numerals 44 and 48. Due to the difference in size between DIS XL-1 and DIS XL-3, mixed substrate reactions produce two high-molecular-weight products differing in size depending on which target DNA segment participated in the reaction. Reaction mixtures that contained integrase are marked '+' and those that did not contain integrase are marked '–' at the top of each lane. Also indicated are pairs of reactions containing either DIS XL-1 (44-mer), DIS XL-3 (48-mer) or a mixture of these two substrates (DIS XL1/3).

derived from DIS XL-1 migrated at this position as well and was therefore indistinguishable from the product derived from the DIS XL-3 substrate (fig. 7). To emphasize this point, integrase was incubated with an equal mixture of the DIS XL-1 and DIS XL-3 substrates. The 21-mer product obtained in this case was indistinguishable in size from the molecule produced in reactions in which each substrate was incubated separately with integrase (fig. 7). The 21-mer product therefore probably does not contain any sequences derived from the host DNA portion of these DNA substrates, consistent with the interpretation suggested earlier that this DNA product is the U5 LTR arm of the dumbbell substrates. The results also indicate that the reactions with DIS XL-1 and DIS XL-3 proceeded best at pH 7.2 versus pH 6.8.

Detection of Putative Intermolecular Reaction Products

In addition to the U5 LTR product of the intramolecular disintegration reaction, incubation of HIV-1 integrase with DIS XL-3 (and DIS XL-1) produced a second radiolabeled DNA species that migrated more slowly than the 48-mer DIS XL-3 substrate (fig. 8). The slowly migrating product obtained with DIS XL-3 appeared to be larger than the analogous product obtained with DIS XL-1. When incubations were done with mixed substrates as before, two slowly migrating DNA products were obtained (fig. 8). Since, they are radiolabeled and since their size varies according to the size of the 3′ portion in the substrate, the high-molecular-weight DNA products appear to contain both 5′ and 3′ portions of the dumbbell substrates DIS XL-1 or DIS XL-3. The fact that two, rather than three, slowly migrating products were observed in the mixed substrate reaction suggests that host DNA portions from separate molecules are not joined together. The high-molecular-weight products may therefore result from the intermolecular transfer of a U5 LTR arm to a molecule of DIS XL-1 or DIS XL-3 in a 5′ joining reaction (see diagram in fig. 8). It was also very clear in this series of experiments that these slowly migrating DNA products were not produced at all in the absence of integrase.

Reactivity of HIV-1 Integrase with a Dumbbell Substrate Containing a 5′ Oligo-dA Tail

We designed another disintegration substrate, DISPOL, which was identical to DIS XL-1 except for an oligo-dA_5 tail which was added in place of the 5′-terminal CA dinucleotide. Integrase was able to catalyze both intra- and intermolecular reactions with this dumbbell substrate, resulting in the production of a fast migrating intramolecular cleavage product and a slowly migrating putative 5′-end joining product, respectively (fig. 9). This result shows that the 5′-terminal AC dinucleotide is not required for the disintegration reaction. Reactions proceeded most efficiently at pH 7.2 and at 37 °C as opposed to pH 6.8 at 30 °C. The intramolecular disintegration product obtained with the DISPOL substrate appeared to be about 24 nucleotides long and migrated more slowly than a 21-mer oligonucleotide marker. An oligo-dA_5 tail in the disintegration product would make it longer by 3 nucleotides than the analogous product derived from DIS XL-1. The intramolecular disintegration product of the DISPOL reaction therefore appears to contain the oligo-dA_5 tail, as expected for the U5 LTR portion of the substrate.

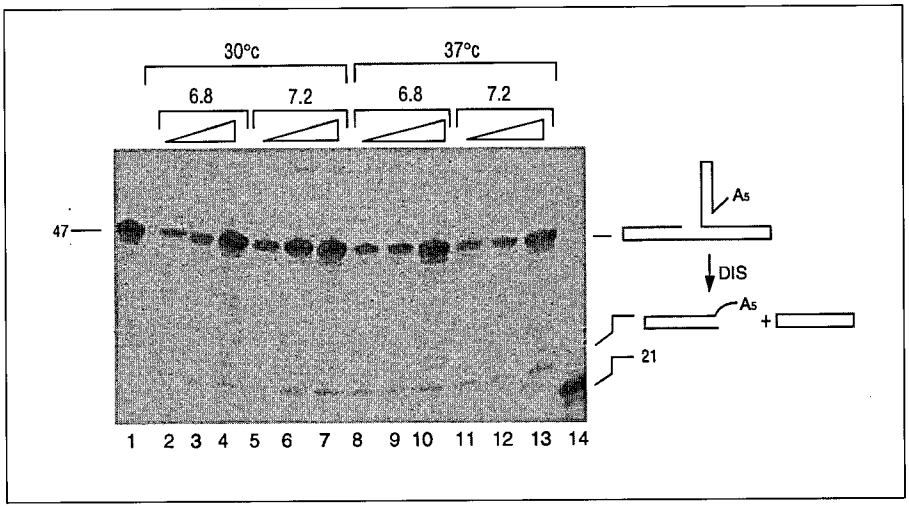

Fig. 9. Reactivity of integrase with a dumbbell substrate containing a 5′ oligo-dA tail. The starting 5′-end-labeled substrate is identical to DIS XL-1 (diagrammed in figure 5) except that the 5′-terminal AC dinucleotide in DIS XL-1 (44-mer) is replaced by an oligo-dA 5-mer. This substrate, referred to in the text as DISPOL, is therefore 47 nucleotides long, as indicated at the left of the autoradiogram. The presumptive mechanism of disintegration with the DISPOL substrate, illustrated at the right, is identical to that with DIS XL-1 except that the released LTR arm is 3 mucleotides longer than for DIS XL-1 because of the 5-mer oligo-dA tail. The LTR arm released from DISPOL (lanes 2–13 in the autoradiogram) therefore migrates more slowly than the 21-mer oligonucleotide marker (lane 14). A reaction mixture in which integrase was omitted was analyzed in lane 1. Inclined plane symbols refer to increasing amounts of DISPOL DNA of 2.5, 5.0 and 10 ng. The pH of each set of reactions and the temperature of incubation are indicated at the top of the figure.

Efficient Detection of Disintegration Products Using a DNA Polymerase Fill-In Reaction

We noticed that U5 LTR disintegration products derived from dumbbell substrates are hairpinned at one end and possess a base-paired 3′ terminus and a 5′ overhang at the other end. In the case of DISPOL we reasoned that it should be possible for the Klenow fragment of *E. coli* DNA polymerase I to copy the 5′-nucleotide oligo-dA 5′ overhang using the base-paired 3′-OH terminus as a primer. Two radioactive DNA products were produced when the disintegration reaction was performed with a nonradioactive DISPOL substrate coupled to a DNA polymerase fill-in reaction in the presence of α-^{32}P-labeled thymidine 5′-triphosphate. The major product of the coupled integrase/DNA polymerase reaction (fig. 10, lanes 6 and 7) comigrated with a marker oligonucleotide of the exact structure and sequence expected for the DNA polymerase fill-in product, i.e. a U5 LTR molecule 30 base pairs long and hairpinned at one end (fig. 10, lane 1). A minor product migrated more slowly than the U5 LTR 30 base pair standard, at the position expected for the DISPOL dumbbell substrate. The latter DNA apparently corresponds to incorporation of radiolabeled TMP at the 3′ terminus of the DISPOL substrate since it was the only product observed when the DISPOL substrate was incubated with Klenow DNA polymerase alone, in the absence of integrase. As a control experiment, the DNA polymerase reaction was performed with a U5 LTR hairpin molecule containing a 5′ oligo-dA$_5$ overhang. The single product of this reaction comigrated with the 30 base pair U5 LTR standard and with the major product of the coupled DNA polymerase/integrase reaction (fig. 10, lane 2).

Next we determined the time course of the coupled integrase/DNA polymerase reaction and compared the results obtained with the Klenow DNA polymerase, an exonuclease minus mutant form of the Klenow enzyme and T4 DNA polymerase. The major DNA species obtained in the coupled integrase/DNA polymerase reaction migrated as a doublet with the 30 base pair U5 LTR fill-in standard (fig. 11). The amount of this DNA increased over a 50-min reaction period with the Klenow DNA polymerase. None of this DNA was detected in the absence of integrase (fig. 11, lane 2). Also, very little, if any radiolabeling of the DISPOL substrate occurred in this experiment. The results for the exonuclease mutant enzyme and the T4 DNA polymerase were essentially identical to those obtained for the Klenow DNA polymerase (fig. 11, lanes 9 and 10, respectively).

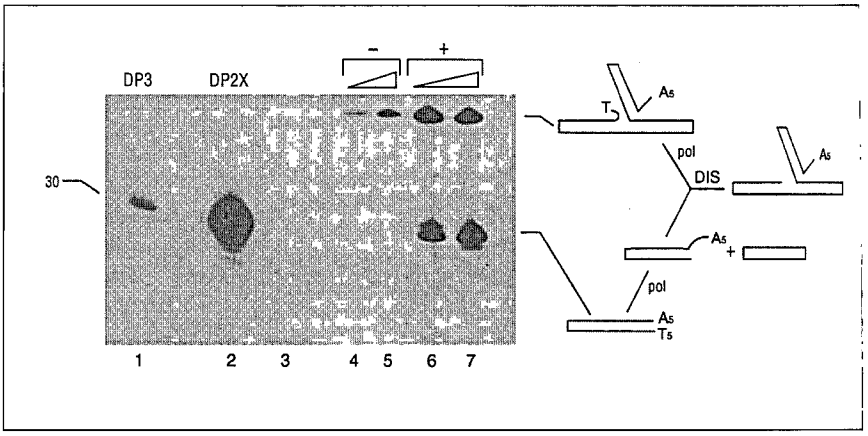

Fig. 10. Detection of disintegration products in a DNA polymerase reaction. Disintegration reactions were conducted with nonradioactive DISPOL DNA in the presence of the *E. coli* DNA polymerase I (Klenow fragment) and α-^{32}P-TTP as described in Materials and Methods. The role of *E. coli* DNA polymerase I (Klenow fragment) in the detection of disintegration products is illustrated to the right of the autoradiogram. Disintegration of the DISPOL substrate by integrase produces a free LTR arm with a recessed 3′ end and a 5′ oligo-dA tail. The reaction conditions are such that the oligo-dA tail is utilized as a template by the DNA polymerase which catalyzes the incorporation of 5 complementary nucleotides into DNA, using the free LTR oligo-dA arm (25-mer) as a DNA substrate. The product of this reaction is therefore a radiolabeled 30-mer hairpin molecule, in which there are 5 dAT base pairs at one end. In order for the radiolabeled 30-mer to be produced under the conditions provided, integrase must release the LTR oligo-dA arm from DISPOL in the disintegration reaction by precise cleavage at the end of the LTR portion of the DISPOL substrate. The upper part of the diagram illustrates the fact that DISPOL DNA can also act as a substrate for *E. coli* DNA polymerase I (Klenow fragment) without the need for integrase. The radiolabeled product of this reaction is a molecule in which a single nucleotide is incorporated at the 3′ end of DISPOL DNA. The position of an authentic 5′-end-labeled 30-mer LTR hairpin of the type described above is indicated at the left of the autoradiogram. A sample of this marker DNA was placed in lane 1, marked DP3. A sample of the free LTR oligo-dA arm that has been incubated with *E. coli* DNA polymerase I (Klenow fragment) under the conditions referred to above, is analyzed in lane 2, marked DP2X. Reactions with DISPOL which did not contain integrase are marked by a '–' and those that did contain integrase are marked by a '+' at the top of each lane. The inclined planes represent increasing concentrations of the DISPOL DNA substrate from 5 to 10 ng.

Discussion

The role of disintegration in the overall scheme of the retroviral integration mechanism remains poorly understood. We have used various dumbbell and Y-substrates to study the requirements for the disintegration reaction in an effort to gain additional insight into this problem. Reactivity with both types of substrate was dependent on Mn^{2+} and displayed a strikingly sharp pH optimum between 6.8 and 7.2. There appeared to be a requirement for the CA dinucleotide at the 3′ end of the LTR and the LTR portion of the disintegration substrate had to be greater than 5 base pairs in length; possibly as many as 10 base pairs were required. Varying the size of the host DNA portion of the dumbbell substrate so that it was composed of either 10 or 12 base pairs had no effect on the extent of the disintegration reaction, in agreement with the results of previous studies [10]. The AC dinucleotide at the 5′ end of the LTR was dispensable and could be replaced with a 5-nucleotide oligo-dA tail without affecting disintegration.

The reaction mechanism appeared to involve the nucleophilic attack of the 3′-OH group from the host DNA portion of the substrate on the phosphodiester bond immediately adjacent to a CA dinucleotide in the viral LTR portion of the substrate. This intramolecular reaction separated the LTR and host DNA portions of the substrate from each other and as such represented a reversal of the 3′-end joining step of integration. When the LTR 5′ end consisted of an oligo-dA tail, *E. coli* DNA polymerase or T4 DNA polymerase were able to completely copy the oligo-dA template, in the presence of TTP as the only nucleotide. The 3′ end of the LTR disintegration product, as liberated by integrase, must therefore be base paired to the template at exactly the beginning of the oligo-dA tail, indicating that nucleophilic attack must occur adjacent to the CA dinucleotide.

As in the present study, other workers who have used dumbbell substrates to study disintegration have observed the production of a discrete high-molecular-weight product, of unknown origin and structure [11]. These

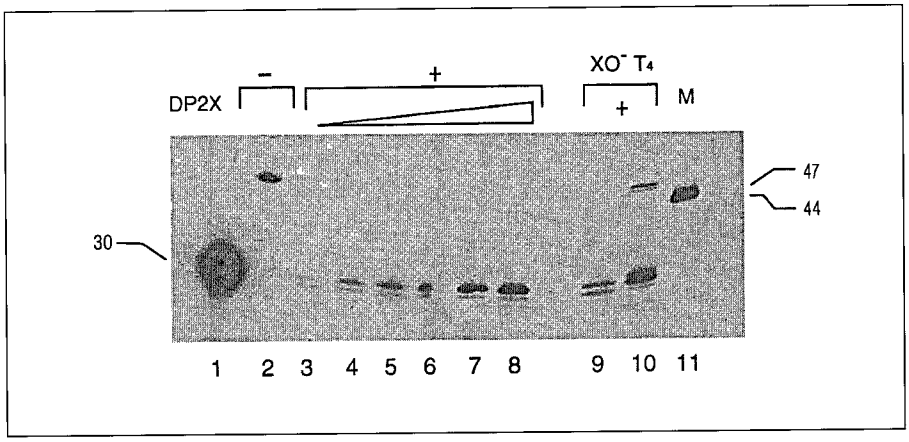

Fig. 11. Time course of the coupled integrase-DNA polymerase reaction and comparison of a wild-type and an exonuclease minus variant of *E. coli* DNA polymerase I (Klenow fragment) and bacteriophage T_4 DNA polymerase. Disintegration reactions with nonradioactive DISPOL DNA were incubated for 30 min at 37°C as described in Materials and Methods. The Klenow fragment of *E. coli* DNA polymerase I was added along with 10 × Klenow buffer and α-^{32}P-TTP and reaction mixtures were incubated for an additional 0, 10, 20, 30, 40 or 50 min (lanes 2–8) at room temperature, as symbolized by the inclined plane and the '+' sign. A reaction mixture without integrase was analyzed in lane 2, designated with a '–' symbol at the top of the lane. Results obtained for the exonuclease minus mutant of *E. coli* DNA polymerase I (Klenow fragment) and T4 DNA polymerase are presented in lanes 9 and 10, marked XO⁻ and T_4, respectively. These samples were incubated for 50 min each. The positions of a DP2X marker DNA applied to lane 1 and of a 5′-end-labeled DIS XL-1 marker (44-mer) applied to lane 11 (marked M) are indicated at the left and right of the autoradiogram, respectively. The expected position of the 47-mer DISPOL substrate is also indicated at the top right of the figure.

workers attributed this product to integration of the LTR arm, released by disintegration, into the original substrate. However, such a reaction would break the dumbbell substrate into two parts at the site of integration, thus causing the appearance of reaction products smaller than the original substrate. Since integration occurs randomly, the reaction products would also be heterogeneous in size. Neither of these expectations is consistent with the observed production of a discrete high-molecular-weight DNA product. Our data showing that the high-molecular-weight product and the LTR arm are produced with identical kinetics argue against this possibility (i.e. reintegration) as well, since the expected lag in the appearance of the high-molecular-weight product is not observed. The fact that a high-molecular-weight product was not observed when integrase was incubated with a dumbbell substrate and a radiolabeled LTR molecule [Garg and Faust, unpubl. data] also argues against this possibility. Since integrase can act in *trans*, by bringing together two substrate molecules to catalyze intermolecular disintegration [7, 8, 38], we considered the possibility that *trans* disintegration between two dumbbell molecules was occurring. The product of this reaction would consist of two host DNA portions linked to one LTR arm. This possibility was ruled out by the results of mixed substrate reactions in which molecules differing in the size of their host DNA portions were used. Joining of a dumbbell substrate molecule to a released unlabeled 22-mer (for DIS XL-1) circular product can be ruled out on the same basis. We therefore suspect that the high-molecular-weight product consists of two LTR arms attached to a host DNA segment. Integrase could accomplish this by bringing two dumbbell molecules together, catalyzing disintegration on one substrate molecule and joining the free LTR arm to the other dumbbell molecule in a concerted manner. We speculate that the joining reaction involves the nucleophilic attack of the 3′-hydroxyl of the recipient dumbbell molecule on the phosphodiester bond joining the 5′-terminal unpaired dinucleotide to the LTR arm as illustrated in figure 8. The mechanism we are proposing for the generation of the high-molecular-weight product therefore constitutes a concerted disintegration and 5′-end joining reaction.

The results of this study do not allow us to arrive at a conclusive role for disintegration in the overall scheme of the integration process as it occurs in vivo. Disintegration may simply be invoked as a way of reversing autointegration that may occur before the preintegration complex reaches the nucleus. Possibly, autointegrants are precursors of the 3′-end joining reaction and disintegration occurs as a prerequisite to and in concert with 3′-end join-

ing. Alternatively, as indicated in the present study, perhaps there is a role for disintegration in relation to 5′-end joining, as others have suggested [9]. Others have shown that integrase can catalyze 5′-end joining, albeit inefficiently [24]. Whatever its role might be in vivo the disintegration activity of integrase may be used as a target for antagonists of integrase that are directed specifically at the D,D,E active site domain of the enzyme, since other domains of integrase are not required for disintegration [2, 12, 22]. Our demonstration that disintegration products can be detected in a DNA polymerase reaction may be useful as a basis for the development of a specific and rapid nucleotide polymerization assay for the measurement of the disintegration function of integrase.

Acknowledgements

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