In Vitro Activities of Purified Visna Virus Integrase

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Although integration generally is considered a critical step in the retrovirus life cycle, it has been reported that visna virus, which causes degenerative neurologic disease in sheep, can productively infect sheep choroid plexus cells without detectable integration. To ascertain whether the integrase (IN) of visna virus is an inherently defective enzyme and to create tools for further study of integration of the phylogenetically related human immunodeficiency virus type 1 (HIV-1), we purified visna virus IN by using a bacterial expression system and applied various in vitro oligonucleotide-based assays to studying this protein. We found that visna virus IN demonstrates the full repertoire of in vitro functions characteristic of retroviral integrases. In particular, visna virus IN exhibits site-specific endonuclease activity following the invariant CA found two nucleotides from the 3' ends of viral DNA (processing activity), joins processed oligonucleotides to various sites on other oligonucleotides (strand transfer or integration activity), and reverses the integration reaction by resolving a complex that mimics one end of viral DNA integrated into host DNA (disintegration activity). In addition, although it has been reported that purified HIV-1 IN cannot specifically nick visna virus DNA ends, purified visna virus IN does specifically process and integrate HIV-1 DNA ends.

In the retrovirus life cycle, a double-stranded DNA copy of the viral RNA genome is inserted into host cell chromosomal DNA in a process called integration. This DNA recombination event is catalyzed by the integrase (IN) protein encoded in the 3' region of the viral *pol* gene. The recent development of oligonucleotide-based in vitro assays for studying purified integrase proteins (7, 9, 25, 27) has expanded greatly our knowledge about this aspect of retrovirus replication. Following completion of reverse transcription, IN produces site-specific cleavages immediately after the conserved CA bases typically found two nucleotides from each 3' end of double-stranded, blunt-ended, linear viral DNA (27). This endonuclease activity of IN, referred to as 3' processing (9, 28), produces the immediate precursor to the integrated provirus (4, 16). IN then catalyzes a one-step transesterification reaction in which the recessed 3' OH groups that were produced by the processing reaction at each end of viral DNA act as nucleophiles and attack phosphodiester bonds in host DNA at the sites of insertion (14). This polynucleotidyltransferase activity of IN, variously referred to as strand transfer, DNA joining, or integration (9, 14, 25), covalently joins the viral DNA 3' ends to 5' phosphate groups of host DNA (4, 14, 16). Cleavage of either strand of host DNA at the sites of integration occur without specificity for DNA sequence but with a spacing characteristic for each retrovirus. Removal of the two nucleotide overhang at each 5' end of viral DNA and filling in of complementary host bases, perhaps by host proteins, result in an integrated provirus which is shortened by two base pairs at each end and flanked by a short duplication of host DNA. In vitro, IN also can catalyze the reverse of the strand transfer reaction to yield the recessed CA-3' OH end of viral DNA, as well as target DNA in which the nick has been sealed (7). This

activity of IN, referred to as disintegration, may or may not have a biological role (7).

Although integration generally is considered a critical step in the retrovirus life cycle, it has been reported that visna virus, a pathogenic retrovirus of sheep, can productively infect sheep choroid plexus cells without detectable integration (18). To ascertain whether the integrase of visna virus is an inherently defective enzyme and to provide tools for comparative studies of integration of the phylogenetically related human immunodeficiency virus type 1 (HIV-1), we purified visna virus IN by using a bacterial expression system and studied it by using various oligonucleotide-based assays. We found that visna virus IN demonstrates the full repertoire of in vitro integrase activities, including 3' processing, strand transfer, and disintegration, and thus can be considered a typical retroviral integrase. In addition, although it has been reported that purified HIV-1 IN cannot specifically nick visna virus DNA ends (31), purified visna virus IN does specifically process and integrate HIV-1 DNA ends.

MATERIALS AND METHODS

Source of visna virus IN coding sequence. Molecular clone 8-5 of Icelandic strain 1514 of visna virus was obtained from Janice Clements, Johns Hopkins University (3). The 281-amino-acid IN protein encoded by this DNA is identical to that of infectious molecular clones (1, 45). Comparisons with other retroviral sequences indicated that visna virus IN is encoded from nucleotide 4164 to a termination codon starting at nucleotide 5006 (3); the predicted amino terminus is a Trp residue following Tyr, consistent with known retroviral protease cleavage sites (43), 11 amino acids before a conserved His-His-Cys-Cys motif found near the amino termini of all known IN proteins (29).

Expression and purification of visna virus IN. Visna virus IN was expressed by using the QIAexpress system type IV construct (Qiagen, Inc., Chatsworth, Calif.), which adds six histidine residues to the N terminus of proteins expressed from the DNA insert. The IN coding region of the visna virus

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genome was amplified by PCR. The 5' primer for PCR, 5'GG AGATCT(ATCGAAGGTAGA)TGGATAGAAAATATTC CCCTAGCAGA3', includes a *Bgl*II restriction site (underlined), followed by sequences coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg in parentheses) and the first 26 nucleotides coding for IN (the Trp codon is in boldface). The 3' primer for PCR, 5'CTAAGTGGAGCTCCTTCCATGC CCATAGTGGCA3', includes an *Sst*I restriction site (underlined) and hybridizes approximately 25 nucleotides downstream of the IN coding region. PCR was conducted in a 100- μ l mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM each dATP, dCTP, dGTP, and TTP, 60 pmol of each primer, 100 ng of clone 8-5 DNA, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). After the mixture was heated to 94°C for 45 s and cooled to 72°C, MgCl₂ was added to 4 mM, and cycling was conducted for 35 cycles of annealing and extension at 65°C for 2 min and denaturation at 94°C for 15 s, followed by a final extension at 65°C for 10 min, utilizing a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Cetus). The 923-bp PCR product was digested with *Bgl*II and *Sst*I and ligated into plasmid pQE-30 (Qiagen) that had been digested with *Bam*HI and *Sst*I. *Escherichia coli* M15[pREP4] (Qiagen) was transformed with the ligation reaction, and colonies resistant to ampicillin and kanamycin were screened for the presence of insert DNA by restriction endonuclease digestion of plasmid DNA, and for production of IN following induction with isopropyl- β -D-thiogalactopyranoside (IPTG), as instructed by the manufacturer.

For large-scale production of IN, an overnight culture grown in 2xYT broth containing 100 μ g of ampicillin per ml and 25 μ g of kanamycin per ml was diluted ~1:50 and grown at 37°C until the optical density at 600 nm was 0.7 to 0.9. IPTG was then added to 2 mM to induce protein expression, and 3 h later the bacteria were harvested by centrifugation and stored at -80°C. To purify IN, the pellet from a 250-ml culture was thawed on ice and suspended in 2.5 ml of sonication buffer (50 mM sodium phosphate [pH 7.6], 10 mM 2-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). Following addition of lysozyme to 1 mg/ml and 30 min of incubation on ice, 1/4 volume of 5 M NaCl was added, and the suspension was sonicated in a cup horn (Heat Systems, Inc., Farmingdale, N.Y.) with seven 1-min bursts separated by 1 min of cooling. The lysate was passed through an 18-gauge needle five times to decrease viscosity and centrifuged at 40,000 $\times$ g and 4°C for 45 min, and the first supernatant was harvested. To extract additional IN, the pellet was suspended in 1.5 ml of fresh sonication buffer, NaCl was added to 1 M, and sonication and centrifugation were performed as before. The second supernatant was added to the first, and the combined solutions were adjusted to 40 mM imidazole by addition of 1/10 volume of sonication buffer-1 M NaCl-440 mM imidazole. This sample was then added to 0.4 ml of washed Ni²⁺-nitrilotriacetic acid agarose (Ni-NTA resin; Qiagen) and stirred for 1 h at 4°C to enhance binding of polyhistidine-tagged proteins. The slurry was poured into a small polypropylene column and allowed to settle for 1 h, after which the unbound material was slowly collected. Pilot purifications indicated that irrelevant proteins were removed from the column by extensive washing with low concentrations of imidazole and that IN began to elute slowly at 80 mM imidazole. Thus, the resin was washed with 8 ml (20 column volumes) of SBNT (sonication buffer with 1 M NaCl and 0.1% Triton X-100) containing 40 mM imidazole (fractions 1 and 2), 18 ml of SBNT containing 60 mM imidazole (fractions 3 to 5), and 0.5 ml of SBNT containing 70 mM imidazole (fraction 6). IN was eluted with SBNT containing 250 mM imidazole, and 0.5-ml fractions were collected.

Purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.8% separation gels and 3% stacking gels (acrylamide-to-methylene-bisacrylamide ratio, 37.5:1). Expressed visna virus IN has a predicted molecular weight of 34,185 (32,330 for the 281 amino acids of IN plus 1,855 for the 16 additional N-terminal amino acids as a result of the cloning scheme, i.e., Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-Ile-Glu-Gly-Arg). In an attempt to remove residual proteins not migrating at the expected position and also to concentrate IN, peak fraction 7 was diluted to 40 mM imidazole, reappplied to a fresh 0.2-ml Ni-NTA column, and washed as before, but eluted with SBNT containing 300 mM imidazole. IN-containing fractions were dialyzed individually three times against 500 volumes of dialysis buffer (50 mM Tris-HCl [pH 7.6], 1 M NaCl, 1 mM dithioerythritol [DTE], 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol); purification conducted with 0.5 M NaCl in column buffers resulted in precipitation of visna virus IN during dialysis, a problem previously encountered with Rous sarcoma virus (RSV) IN (26). The dialyzed product was adjusted to 40% glycerol by addition of 1/2 volume of 100% glycerol, yielding a protein storage buffer of 33 mM Tris-HCl (pH 7.6), 0.67 M NaCl, 0.7 mM DTE, 0.07 mM EDTA, 0.07% Triton X-100, and 40% glycerol; aliquots were stored at -20 or -80°C. Protein concentration was measured by the Bradford method with bovine gamma globulin as standards (Bio-Rad, Hercules, Calif.).

Oligonucleotides. Oligodeoxynucleotides used in these studies typically were gel purified following synthesis. 5'-end-labeled sequences were prepared by using [γ -³²P]ATP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.) and T4 polynucleotide kinase (Promega, Madison, Wis.). For labeling near the 3' end of the visna virus U3 negative-strand oligonucleotide, unlabeled visna virus U3 processed substrate (Fig. 1A) was filled in with [α -³²P]dGTP (800 Ci/mmol; New England Nuclear) and nonradioactive TTP by incubation with the Klenow fragment of DNA polymerase I (Promega) at 37°C for 30 to 60 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 6.5 mM MgCl₂, 1 mM DTE, and 32 μ g of bovine serum albumin per ml. All labeled oligomers were purified on 20% polyacrylamide-7 M urea denaturing gels, visualized by autoradiography, eluted from gel slices in 0.5 M ammonium acetate-1 mM EDTA at 37°C, concentrated by binding to C18 resin minicolumns (Waters Chromatography, Milford, Mass.), eluted with 25% acetonitrile, and evaporated to dryness. Specific activities of radiolabeled oligonucleotides, as ascertained by the DE81 filter binding method (39), were 10⁶ to 10⁷ cpm/pmol. Sequence-specific markers for gel analysis were produced by the 3'-to-5' exonuclease activity of snake venom phosphodiesterase (Sigma, St. Louis, Mo.) on 5'-radiolabeled oligonucleotides as previously described (27).

Endonuclease (processing) assays. For reactions requiring double-stranded DNA substrates, unlabeled oligonucleotide was added to 0.03 pmol of labeled oligomer to yield a total of 0.5 pmol (specific activity, ~0.5 $\times$ 10⁶ cpm/pmol) and mixed with 2 pmol of complementary oligonucleotide in 6 μ l of water. Annealing was achieved by heating at 95°C for 5 min followed by incubation at 37°C for 30 min and 4°C for at least 10 min. Buffers, protein, and divalent cations were then added, in that order. Standard 10- μ l reaction mixtures contained 0.5 pmol of single- or double-stranded DNA, 25 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 1 μ l of IN (~40 pmol) or protein storage buffer, and 10 mM MnCl₂. Reaction mixtures were incubated for 60 min at 37°C, and then reactions were stopped by addition of 10 μ l of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and

FIG. 1. Synthetic oligonucleotide substrates and integrase assays. (A) Oligonucleotide substrates derived from the U3 or U5 terminus of visna virus or HIV-1 are shown beneath a schematic representation of the linear form of viral DNA in which each long terminal repeat (LTR) consists of U3, R, and U5 regions. In all cases, the positive (+) strand of viral DNA is shown above the negative (−) strand. The invariant CA dinucleotides in strands that get nicked by IN are in boldface, and asterisks represent ^{32}P groups. Sites of nicking for the blunt-ended visna virus U3 and U5 substrates are indicated by arrows. Two nucleotides following the conserved CA are missing from the visna virus U5 and U3 processed substrates. Base substitutions of the conserved CA (and its complement) are shown in lowercase for the visna virus U5 altered substrate. (B) In vitro integrase assays are shown schematically for the visna virus U5 substrate. With blunt-ended DNA, the endonuclease activity of IN produces a site-specific nick after the conserved CA, yielding a radioactive oligomer two nucleotides shorter than substrate. With processed substrates, the DNA joining (integration, strand transfer) activity of IN inserts the recessed CA terminus into sites within other oligonucleotides (shown as dotted lines), yielding labeled integration products longer than substrate; for clarity of presentation, the donor DNA representation was rotated. Shorter labeled oligomers (secondary cleavage sites) also are produced in the case shown because the labeled strand of a processed oligonucleotide acts as target; shorter products would not be labeled when the other strand acts as the target. Both DNA nicking and joining can occur sequentially in one assay. IN also can catalyze the reverse of the DNA joining reaction, termed disintegration.

heating at 95°C for 5 min. Terminated reactions were kept on ice or stored at −20°C. For gel analysis, aliquots were heated, loaded onto 20% polyacrylamide (acrylamide-to-methylenebisacrylamide ratio, 19:1)–7 M urea denaturing gels, and then electrophoresed at 55 W until the bromophenol blue dye had migrated 23 cm. Wet gels were autoradiographed with Kodak X-Omat film at −80°C. Specific cleavage was demonstrated by the appearance of a new radiolabeled oligomer two nucleotides shorter than the starting substrate (Fig. 1B).

Strand transfer (integration) assays. To assay directly for DNA joining activity, duplex oligonucleotides in which the 5'-labeled strand was missing two nucleotides from the 3' end, and thus presented a recessed CA-3' OH group as in the immediate precursor to the integrated provirus, were prepared and used under conditions identical to those for endonuclease assays. Strand transfer was demonstrated by the appearance of radiolabeled products longer than substrate length (Fig. 1B).

Disintegration assays. The reversal of strand transfer assays were done under conditions identical to those described above except that 0.02 pmol (10^5 cpm) of a Y-shaped complex of four oligonucleotides was used as the substrate. This complex was designed to mimic the immediate integration product of the visna virus U5 DNA end into a nonviral DNA sequence (Fig. 1B), and care was taken in the design of host DNA surrogate sequences to remove all competing structures, including inverted repeats, as well as fortuitous CA dinucleotides. Complexes were made by radiolabeling the 5' end of oligomer 1 or oligomer 2 (see Fig. 9A), gel purifying each of these as described above, and annealing each with a fivefold molar excess of the other three oligomers in 0.1 M NaCl–1 mM EDTA–10 mM Tris-HCl (pH 7.5). Because the estimated melting temperatures for the three predicted duplexes were 50 to 54°C, annealing was performed by heating at 95°C for 3 min,

60°C for 5 min, 55°C for 20 min, 50°C for 20 min, 45°C for 20 min, 37°C for 10 min, and 4°C overnight; prolonged incubation at 4°C was found to enhance complex formation. The Y-shaped complex was separated from the bulk of unannealed oligomers by electrophoresis on a native 15% polyacrylamide gel; the position of the complex was confirmed by sequentially mixing all combinations of oligomers that could anneal and loading these in adjacent lanes. As expected, this complex migrated most slowly and identically whether oligomer 1 or oligomer 2 contained the 5' radiolabel. The integrity of the complex was maximized by elution from gel slices in 0.5 M ammonium acetate–10 mM magnesium acetate at 30°C overnight, followed by concentration with Centricon-10 filters (Amicon) and washing with 25 volumes of 0.1 M NaCl–1 mM EDTA–10 mM Tris-HCl (pH 7.5) as described by Chow et al. (7).

Quantification of results. Where indicated, radioactivity of bands in wet gels was quantified with a Betascope (Betagen, Waltham, Mass.), and intensities of bands on autoradiograms were measured with a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.) using software from Protein Database, Inc. (Huntington, N.Y.).

RESULTS

Purification of visna virus IN. Visna virus IN was expressed with a short N-terminal extension including six histidine residues, facilitating rapid and efficient protein purification by metal affinity chromatography with a nickel-chelating resin. As seen in Fig. 2, a protein with an apparent molecular mass of 35 kDa was induced by addition of IPTG (lanes b and c); densitometric scanning of a Coomassie blue-stained gel indicated that ~10% of the total protein migrated at this position.

FIG. 2. SDS-PAGE demonstrating purification of visna virus IN. Aliquots from the protein purification were heated in sample buffer and separated by SDS-PAGE, and the gel was stained with Coomassie blue. Lanes: a, molecular mass markers (sizes are shown in kilodaltons at the left); b and c, sample of uninduced and induced, respectively, bacterial pellets at time of culture; d, 1 μ l of induced bacterial lysate at time of purification; e, sample of residual pellet after two extractions; f and g, 2- μ l aliquots of supernatants from first and second extractions, respectively, of induced bacterial lysate; h, 5 μ l of material applied to Ni-NTA resin; i, 5 μ l of column pass-through; 1 to 6, 16- μ l aliquots of wash fractions that contained increasing amounts of imidazole (Imid), as indicated; 7 to 12, 5- μ l aliquots of fractions eluting off the column with 250 mM imidazole. The arrow indicates an $\sim$ 35-kDa protein induced by addition of IPTG and purified from the Ni-NTA resin.

Although much of this protein remained in the insoluble pellet (lane e), a significant amount was solubilized by two extractions with high salt (lanes f and g). This band represented $\sim$ 3% of the total protein loaded onto the column (lane h), and at least two-thirds of this material bound to the resin (lane i). Following extensive washing with low concentrations of imidazole, most of this protein eluted in the first column volume when 250 mM imidazole was applied to the resin. Gel analysis of peak fraction 7 demonstrated that the 35-kDa IN was purified to near homogeneity, with a small amount of faster-migrating bands (perhaps premature termination or breakdown products) and a trace amount of much slower bands (migrating approximately at the position of dimeric IN). Two lines of evidence suggest that bands migrating at positions other than 35 kDa were specifically related to the hexahistidine-tagged visna virus IN. Dilution of peak fraction 7 to 40 mM imidazole and application to a second Ni-NTA column, under conditions in which there would be less nonspecific competition for resin binding sites, followed by extensive washing and elution with 300 mM imidazole resulted in a peak fraction that appeared identical in composition to the input fraction 7 by SDS-PAGE (not shown). In addition, mock purification from bacteria transformed with the parental plasmid vector pQE-30, from which IPTG failed to induce a 35-kDa protein, yielded no protein bands from an identical imidazole gradient (not shown). The final visna virus IN product in protein storage buffer had a concentration of 1.4 mg/ml. Although a factor Xa cleavage site was engineered into this protein between the hexahistidine tag and the authentic N terminus of IN, removal of the extra residues proved to be very inefficient. At best, 50% of the protein could be cleaved with factor Xa by incubation for 40 h at room temperature (data not shown). Thus, as others have done with integrases from murine leukemia virus (MLV) (22), HIV-1 (6, 11, 12), and human

foamy virus (37), all experiments were conducted with the polyhistidine-tagged protein.

Demonstration of specific endonucleolytic 3' processing. As originally described for avian myeloblastosis virus (AMV) IN (27), duplex oligonucleotides which mimic the blunt-ended linear viral DNA integration substrate were presented to purified visna virus IN, and specific cleavage after the invariant CA near the 3' end was indicated by shortening of the 5'-radiolabeled strand by two nucleotides (Fig. 1B). By using visna virus U5 duplex 18-mers with the 5' label on the positive strand (Fig. 1A), a prominent and specific product of 16 nucleotides was produced during a 75-min incubation with IN in the presence of Mn^{2+} (Fig. 3, open arrow). Cleavage was demonstrated across a range of Mn^{2+} concentrations (results for 1 to 15 mM are shown in Fig. 3, but concentrations up to at least 30 mM supported cleavage), with maximum activity at 10 mM Mn^{2+} . No cleavage was evident if IN was omitted from the reaction (Fig. 3, — lane). Similar results were obtained when the U3 duplex 18-mers were used as the substrate (data not shown). The parallel fraction obtained from the mock purification and handled similarly had no activity in this assay (not shown).

There was an absolute requirement for Mn^{2+} as the divalent cation in processing assays; visna virus IN was not active if Mn^{2+} was omitted or if $MgCl_2$ (range, 1 to 50 mM), $CaCl_2$ (range, 0.2 to 20 mM), or $ZnCl_2$ (range, 0.2 to 20 mM) was used in reaction mixtures that were incubated for 2 h (data not shown). Cleavage activity was similar whether DTE, dithiothreitol, or 2-mercaptoethanol was used as the reducing agent (range of 3 to 30 mM tested for each) or if reducing agents were omitted from reactions (data not shown), although it should be noted that the protein storage buffer contained a low level of reducing agent. Specific DNA nicking was maximal at pH 8.0 to 8.3 but also occurred at pH 7.0. Nicking was greatest

FIG. 3. Endonuclease activity of visna virus IN. The visna virus U5 duplex substrate was incubated without IN (– lane, with 3 mM $MnCl_2$) or with IN in the presence of the indicated concentrations (millimolar) of Mn^{2+} and analyzed as described in Materials and Methods. Reactions differed slightly from standard conditions in that there were 1 pmol of duplex substrate and 3 mM DTE as the reducing agent, and incubations were for 75 min. As shown schematically at the bottom, nicking (arrow) after the conserved CA (boldface) two nucleotides from the 3' end of the 5'-labeled strand (*) converts the 18-mer substrate (closed arrow) to a 16-mer product (open arrow). No major bands were evident in reaction lanes below the position of 10-mers with this radiographic exposure. M1, sequence-specific oligonucleotide ladder produced from the 5'-labeled visna virus U5 positive strand; M2, mixture of M1 with reaction shown in the – lane, demonstrating shifts in migration for this ladder when the complementary strand was present (see Results).

at 37 to 41°C but was easily detected at 25 and 30°C. A time course demonstrated that specific cleavage was evident at the earliest time point tested (1 min) and reached a plateau between 30 and 90 min (Fig. 4A). Addition of NaCl and KCl to reactions inhibited cleavage activity in a dose-dependent manner, whereas dimethyl sulfoxide, bovine serum albumin, glycerol, and ATP had insignificant effects at the doses tested (Table 1). Imidazole at a final concentration of 30 mM diminished activity, underscoring the necessity of dialyzing imidazole away from the purified protein. For subsequent experiments, 10 mM $MnCl_2$, 10 mM dithiothreitol, pH 8.0, 37°C, and 60 min of incubation were chosen as standard reaction conditions.

A curious finding in initial experiments with the U5 duplex substrate was that neither the 18-mer substrate nor the 16-mer cleavage product migrated precisely with bands of the snake venom phosphodiesterase ladder produced from the 5'- ^{32}P -labeled U5 positive strand (Fig. 3, lane M1). Mixing a completed endonuclease assay (lacking IN) with the markers demonstrated that this difference in migration was a gel artifact that ultimately was traced to the presence of complementary unlabeled U5 negative-strand oligomers in the reaction (Fig. 3, lane M2). Experiments demonstrated that the shift upward of these bands was not due to a mass effect but was due to a specific requirement for the complementary strand, despite the denaturing conditions used for sample handling and electrophoresis. A similar phenomenon was observed with all oligomers in this report derived from the visna virus U5 terminus (26); the high GC content of this sequence may partially explain these findings.

Comigration of the 16-mers produced by IN-mediated cleavage of duplex U5 18-mers with the snake venom-produced 16-mers (when unlabeled complementary strand was added to the markers; Fig. 3, lane M2) strongly suggested that visna virus IN had produced 3' OH groups at sites of nicking, as do other integrases (9, 27, 42, 52). Nonetheless, given the anomalous electrophoretic behavior described above, experiments were conducted to test formally whether visna virus IN does act as an endonuclease to produce 3' OH and 5' phosphate groups. To this end, the visna virus U3 duplex substrate (Fig. 1A) was cleaved by IN, and the 16-mer products were gel purified, reannealed to U3 positive-strand 18-mers, and used as primers for the Klenow fragment of DNA polymerase I in the presence of dGTP. As seen in Fig. 5A, the 16-mers (lane 2) did act as primers for Klenow and were extended to 17-mers (lane 3), proving that they had terminal 3' OH groups.

To test whether IN acts as an endonuclease rather than an exonuclease, we constructed a visna U3 negative-strand 18-mer that contained a radiolabel between the invariant CA and the terminal GT (i.e., 5'GTTCTCTGTCCTGACA[^{32}P]GT3'). This oligomer was produced by repair synthesis of unlabeled visna virus U3 processed substrate (Fig. 1A) with [α - ^{32}P]dGTP and nonradioactive TTP. The resulting labeled 18-mers were gel purified and reannealed to U3 positive-strand 18-mers to form a duplex substrate for IN (Fig. 5B). As seen in Fig. 5C, IN cleaved the 18-mers (lane 2) to produce labeled dinucleotides but no detectable mononucleotides (lane 3), proving that IN acts as a DNA endonuclease. As markers for the dinucleotide product, we took advantage of the fact that the snake venom phosphodiesterase ladder produced from 5'- ^{32}P -labeled visna virus U3 negative-strand 18-mers (lane 1) fortuitously included the dinucleotide 5' [^{32}P]-GpT-OH, precisely the expected product for this experiment. Indeed, if IN had acted as an exonuclease, the radiolabel in lane 3 would have been released as 5' [^{32}P]-G-OH, which also is included in the marker lane. Note that the 18-mers in lane 1 migrated faster than uncleaved substrate in lane 2 because the substrate 18-mers do not have 5' phosphate groups.

Treatment of an aliquot of the completed endonuclease assay shown in lane 3 (Fig. 5C) with calf intestine alkaline phosphatase abolished the radioactivity at the dinucleotide position (Fig. 5C, lane 4), confirming that IN had produced a free 5' phosphate group at the site of nicking. The other major bands seen in lanes 3 and 4 (Fig. 5C) presumably correspond to the glycerol-dinucleotide product (form I) and cyclic dinucleotide product (form II) described by others for integrases from HIV-1 (14, 52) and HIV-2 (52) when substrates radiolabeled near the 3' end were used. To form these products, glycerol or the 3' end of the oligonucleotide, rather than water, provides the OH group for nucleophilic attack at the site of DNA cleavage (14, 52). In support of this interpretation, addition of extra glycerol to the endonuclease assay yielded an increase in the intensity of form I (Fig. 5C, lane 5). As expected, the radiolabels in these products were not susceptible to phosphatase treatment (lane 4). Similar to HIV integrases, visna virus IN thus catalyzes a more general alcoholysis, in addition to hydrolysis, when performing the phosphoryl transfer of the 3' processing reaction (52). Taken together, these results prove that visna virus IN acts as a site-specific endonuclease to produce 3' OH and 5' phosphate groups two nucleotides from the 3' ends of sequences representing the termini of visna DNA.

Demonstration of strand transfer (integration) activity. In the endonuclease assay shown in Fig. 4A, products greater than substrate length were evident at the later time points; these bands were easier to detect with a longer radiographic

FIG. 4. Time course of cleavage and integration reaction with visna virus U5 substrate. Aliquots of a standard assay were removed at the time points indicated, added to loading buffer, and heated to stop the reaction. Samples were analyzed by denaturing gel electrophoresis, and counts per minute were quantified by Betascope counting with correction to the 0-min time point. (A) Autoradiogram exposed for 16 h with one intensifying screen; for each time point, the percent specific cleavage (shown beneath lanes) was calculated as (cpm of 16-mer product + cpm of integration products)/(total cpm in lane). (B) Same gel exposed for 5 days with two screens; for each time point, percent integration products was calculated as (cpm of integration products)/(total cpm in lane). Fewer counts per minute were loaded onto the gel for the 120-min sample; similar experiments indicated that plateau values were maintained at even longer times. A sequence-specific nucleotide ladder is placed next to the final lane in panel B to identify secondary cleavage sites. P, position of integration products; S, position of substrate; -2, position of 16-mer cleavage products.

exposure (Fig. 4B). Analogous products for integrases from MLV (9), AMV (25), and HIV-1 (5) have been shown to represent insertion of the processed 3' end of radiolabeled oligonucleotides, produced by the nicking activity of IN, into various sites on other oligonucleotides that act as surrogates for host DNA in reactions that model integration (Fig. 1B). These products were evident after 3 min of incubation with visna virus IN and increased in parallel to the cleavage product (Fig. 4B). When the reaction reached a plateau and almost 50% of substrate had been specifically cleaved, ~3 to 4% was integrated.

The apparent lag between appearance of cleavage products (Fig. 4A, 1 min) and integration products (Fig. 4B, 3 min) suggested a product-substrate ordered relationship in which processed 16-mers must be formed to be inserted into other oligomers. Evidence in support of this notion is shown in Fig. 6, for which visna virus U3 substrates were used. With blunt-ended substrates (Fig. 6A), cleavage was evident after 1 min of incubation with visna virus IN, but integration products were not detected until 3 min of incubation. When product formation reached a plateau at ~40 min of incubation (not shown), ~65% of the substrate had been specifically cleaved and

TABLE 1. Effects of additives on 3' processing reaction

Additive (final concn ^a)	Specific cleavage ^b
None	1.00 ^c
NaCl	
50 mM	0.78
100 mM	0.23
150 mM	0.11
KCl	
50 mM	0.69
100 mM	0.22
150 mM	0.11
Dimethyl sulfoxide (10%)	0.90
Bovine serum albumin (0.1 mg/ml)	0.80
Glycerol (10%)	0.98
ATP (1 mM)	0.95
Imidazole (30 mM)	0.60

^a For NaCl, an additional 67 mM was provided by the enzyme.

^b Visna virus U5 duplex substrate was incubated with IN under standard conditions and analyzed by denaturing gel electrophoresis, and counts per minute were quantified by Betascope counting. Specific cleavage was calculated as (cpm of 16-mer product + cpm of integration products)/(total cpm in lane). Data are representative of two experiments.

^c Data normalized to reaction without additive, which had 47% specific cleavage.

FIG. 5. Proof that IN acts as endonuclease to produce 3' OH and 5' phosphate groups. (A) The 16-mer products of IN-mediated cleavage of the visna virus U3 substrate were gel purified, reannealed with unlabeled visna virus U3 positive-strand 18-mers, and used as primers for the Klenow fragment of DNA polymerase I in the presence of 0.1 mM dGTP (see Materials and Methods for reaction conditions); analysis was by denaturing gel electrophoresis and autoradiography. Extension of 16-mer cleavage products (lane 2) to 17-mers (lane 3) proves the existence of 3' OH groups after cleavage by IN. Lane 1, sequence-specific oligonucleotide ladder produced from 5'-labeled visna virus U3 negative strand as markers. (B) Schematic representation of substrate derived from the visna virus U3 terminus containing a ^{32}P label (*) following the conserved CA (boldface) at the site of nicking (arrow) and used for the experiment shown in panel C. (C) The substrate shown in panel B was incubated without IN (lane 2) or with IN (lane 3) under standard conditions and analyzed as described in Materials and Methods. Production of a labeled dinucleotide (lane 3) proves that IN acted as an endonuclease. Before addition of loading buffer, an aliquot of the reaction shown in lane 3 was treated with calf intestine alkaline phosphatase (CIP) in 10 mM Tris-HCl (pH 8.3)–1 mM MgCl_2 –1 mM ZnCl_2 for 15 min at 37°C and 45 min at 55°C and then subjected to gel analysis. Disappearance of the signal from the dinucleotide position (lane 4) proves that IN produced 5' phosphate groups at sites of nicking. The reaction in lane 5 contained an extra 10% glycerol (final concentration, 14%). Lane 1 shows a snake venom phosphodiesterase ladder produced from the 5'-labeled U3 negative strand; only the 18-mer (5' phosphorylated substrate), 2-mer (^{32}P -GpT-OH), and 1-mer (^{32}P -G-OH) positions are relevant to this experiment. Form I, presumed cleavage product in which glycerol acted as nucleophile in the presence of IN and became attached to the dinucleotide released from the substrate; form II, presumed cleavage product in which the 3' OH of substrate DNA acted as a nucleophile in the presence of IN to yield a cyclic dinucleotide (14, 52).

~14% was integrated. In contrast, when the processing step was bypassed by providing the U3 processed substrate (Fig. 1A), integration products were evident as early as 1 min (Fig. 6B) and ~18% of substrate ultimately became integrated (not shown). With use of preprocessed duplex substrates, optimal conditions for integration were found to be identical to those for IN-mediated cleavage (data not shown). In particular, omission or choice of reducing agent was not important for

FIG. 6. Early time course of cleavage and integration reactions with blunt-ended or processed visna U3 substrates. Aliquots of standard reactions were removed at the time points indicated and analyzed as described in the legend to Fig. 4. Blunt-ended visna virus U3 18-mers were used as the substrate for the experiment in panel A, and a preprocessed substrate was used for panel B; substrates are shown schematically below the autoradiograms with conventions as defined in the legend to Fig. 3 except that arrows denote the site of nicking and/or joining. The band migrating just behind substrate in panel A is occasionally seen on gels and represents either a contaminant or an artifact that is independent of the presence of IN (see Fig. 8). Lane M, sequence-specific oligonucleotide ladder produced from 5'-labeled visna virus U3 negative strand; P, position of integration products; double-headed arrow, position of 16-mer product (A) or substrate (B).

activity, and no activity was evident when Mg^{2+} , Ca^{2+} , or Zn^{2+} replaced Mn^{2+} as the divalent cation. The parallel fraction obtained from the mock purification with the parental plasmid had no activity in this assay (not shown).

Cleavages that are coupled to the integration reaction (the secondary cleavage sites described in the legend to Fig. 1B) also are evident in Fig. 4B and 6B as the minor bands migrating faster than the major processed 16-mers. Apparently almost any site can be used for insertion, since only positions 1, 2, and 3 were not sites of secondary cleavage (Fig. 4B; positions 1 to 4 on the lower part of the gel are not shown). Note that there is a characteristic pattern of preferred sites that is a function of the oligonucleotide sequence (compare secondary bands in Fig. 4B and 6B). Similar results have been noted by others and found to be a function of the target DNA sequence (20, 33).

Possible structural requirements for processing and integration activities. HIV-1 IN (11) and MLV IN (9) are reported to lack specific cleavage activity on single-stranded oligonucleotide substrates. However, AMV IN was able to specifically nick the RSV U5 positive strand even when that strand was presented without its complementary oligonucleotide, although it could not cleave single RSV U3 negative strands (27). To examine whether visna virus IN has a structural requirement for susceptible DNA, we tested the visna virus U5

FIG. 7. Activity on single oligonucleotide versus duplex substrates. Oligonucleotides representing the strands indicated below the panels were 5' labeled and used as ostensibly single-stranded substrates (ss lanes), or after annealing to their complementary oligonucleotides to produce double-stranded substrates (ds lanes), for reactions lacking (– lanes) or containing IN under standard conditions (except that reaction mixtures for panel C were incubated 90 min). M1, sequence-specific oligonucleotide ladder produced from 5'-labeled visna U5 positive (+) strand; M2, mixture of M1 with unlabeled U5 negative (–)-strand oligomer; M, sequence-specific oligonucleotide ladders for the other 5'-labeled oligomers. Arrows denote positions of cleavage products two nucleotides shorter than substrates. On longer radiographic exposures, integration products were detected for all duplex substrates but for no single oligonucleotide substrates. Single oligomer substrates were tested at least twice with similar results.

positive strand and U3 negative strand as substrates in the absence of their complementary oligomers. Surprisingly, both substrates were specifically cleaved by visna virus IN two nucleotides from the 3' end (Fig. 7A and B). Note that two marker lanes were needed for the visna virus U5 substrates (Fig. 7A), the difference being the absence (lane M1) or presence (lane M2) of the complementary negative strand to the labeled U5 positive strand, as discussed earlier. Although integration products were detected in these experiments with duplex substrates, integration products never were observed with the single oligonucleotide substrates, even on long radiographic exposures.

To determine whether any duplex oligonucleotide that is specifically cleaved by visna IN also can be cleaved when presented without its complementary strand, we sought other substrates that were susceptible to cleavage by visna virus IN. We first found that 18-mers derived from the termini of the HIV-1 U5 positive strand and HIV-1 U3 negative strand (Fig. 1A), when annealed with their complements, were specifically cleaved after the invariant CA by visna virus IN (Fig. 7C and D). In contrast, when not annealed with their complements, these oligonucleotides were not active substrates for the specific cleavage activity of visna virus IN. Thus, not all susceptible base-paired oligonucleotides remain active for visna virus IN in the absence of their complementary strands. As with the RSV U5 positive-strand sequence, the susceptibility of visna virus terminal DNA sequences to cleavage by IN in the absence of their complementary oligomers may be due to base pairing by these sequences (see Discussion).

Sequence requirements for processing and integration activities. Since the first report with AMV IN, it has been known that integrases can tolerate some variability in substrate DNA sequence, although bases closest to the cleavage site, especially the invariant CA, are most critical for susceptibility (5, 27, 31,

33, 42, 51). Tolerance for different sequences by visna virus IN is immediately underscored by its activity on both visna virus U5 and visna virus U3 sequences. As shown above, visna virus IN also exhibited specific cleavage activity on duplex oligonucleotides derived from either terminus of HIV-1 (Fig. 7C and D); longer radiographic exposures demonstrated that visna virus IN also integrated these substrates into other oligomers. Activity for visna virus IN on these sequences was somewhat unexpected given that HIV-1 IN was reported to be inactive on the visna virus U5 sequence (31).

We also tested a series of DNA substrates that do not represent a retroviral DNA terminus for susceptibility to visna virus IN. For convenience, we used the same duplexes as those which demonstrated site-specific cleavage by visna virus IN, but placed the 5' radiolabel on the strand that does not contain the invariant CA. No nicking or only a slight amount of nicking of these strands was detected, sometimes including sites two nucleotides from the 3' ends (Fig. 8, first four sets of lanes, arrowheads). However, we never observed the intensity or selectivity for this site as was seen with the authentic visna DNA ends or even with the nonhomologous but authentic HIV-1 DNA ends (Fig. 7). It could be argued that IN was monopolized by the biologically relevant ends of these duplexes for frequent cleavages that would be invisible to these assays because of the placement of the labels. Thus, we also tested duplex oligonucleotides which were predicted to be a poor substrate at both ends. For this purpose, we synthesized 18-mers that represent the visna virus U5 terminus but with the conserved CA changed to a TC, along with its complementary substitution (visna virus U5 altered; Fig. 1A), and either strand was labeled at the 5' end before annealing. As with three of the first four substrates shown in Fig. 8, some cleavage activity was detected on these duplex substrates, including sites two nucleotides from the 3' ends (Fig. 8, last two sets of lanes,

FIG. 8. Activity on duplex substrates with alternative sequences. Oligonucleotides representing the strands indicated (negative [-] and positive [+]; see Fig. 1A) were 5' labeled, annealed to their complementary sequences, and used as substrates for reactions lacking (- lanes) or containing IN under standard conditions except that incubations were for 90 min. Bands migrating just behind substrates in some - lanes are either contaminants or gel artifacts and are not associated with larger integration products. The band migrating just ahead of substrate in the last - lane represents a contaminant that appears to be susceptible to cleavage by IN. Sequence-specific oligonucleotide ladders for each substrate are shown in lanes M; all visna virus U5-derived ladders were spiked with unlabeled complementary oligomers before loading, although for the first set of lanes, marker bands shifted more completely than did reaction bands. Arrowheads denote positions where 16-mer cleavage products would be expected. Larger products were detected for the first three substrates with long radiographic exposures of similar gels. All substrates were tested at least twice with similar results.

arrowheads). However, the markedly diminished activity compared with authentic retroviral terminal sequences now could not be explained by IN being occupied cleaving the unlabeled strand.

Taken together, the results described above indicate that visna virus IN maintains a high, but not absolute, degree of sequence specificity for cleavage near the 3' ends of linear DNA. Additional evidence that placement of nicks two nucleotides from DNA ends is not the sole driving force in specificity comes from an experiment using a substrate that had an extra nucleotide added to the 3' end of the visna virus U3 negative strand; in this case, specific cleavage by IN was detected after the CA which was three nucleotides from the end (data not shown). Sequence specificity for the DNA joining activity of visna virus IN also was not absolute, since long exposures of autoradiograms similar to that shown in Fig. 8 demonstrated products greater than substrate length for three of these duplexes: visna virus U5 labeled on the negative strand (very difficult to detect), visna virus U3 labeled on the positive strand, and HIV-1 U5 labeled on the negative strand.

Demonstration of disintegration activity. As originally described for HIV-1 IN (7), visna virus IN was able to resolve a Y-shaped complex consisting of four annealed oligonucleotides designed to mimic the immediate product of one end of visna virus DNA integrated into nonviral DNA (Fig. 1B and 9A). When this complex contained a 5' 32 P label on oligomer 1, IN cleaved after the CA near the middle of the labeled 31-mer to produce a labeled 16-mer (Fig. 9B, lane 2, open arrow). Concomitant with this activity, IN sealed the nick in the surrogate target DNA of the Y-shaped complex. This fact was demonstrated by using as the substrate a complex that was

FIG. 9. Demonstration of disintegration activity. (A) Schematic representation (7) of the four-nucleotide Y-shaped complex that represents the immediate integration product of the visna virus U5 DNA end into host DNA and used as the substrate for experiments shown in panel B. Oligomer 1 is the visna virus U5 positive strand without the final 2 nucleotides but extended by 15 irrelevant nucleotides. Oligomers 2 and 3 are irrelevant 16- and 31-mers, respectively. Oligomer 4 is identical to the visna virus U5 negative-strand 18-mer. (B) Y-shaped complexes 5' radiolabeled on oligomer 1 (*1, lanes 1 and 2) or on oligomer 2 (*2, lanes 3 and 4) were incubated with the parallel fraction from a control purification (lanes 1 and 3) or with visna virus IN (lanes 2 and 4) and analyzed as described in Materials and Methods. Nicking after the CA in labeled oligomer 1 converts the 31-mer to a 16-mer product (open arrow, lane 2). Ligation of labeled oligomer 2 to the 15 nucleotides released from the 3' end of oligomer 1 converts the 16-mer to a 31-mer product (closed arrow, lane 4).

5' 32 P labeled on oligomer 2. In this case, IN acted to convert the labeled 16-mer into a 31-mer (Fig. 9B, lane 4, closed arrow), presumably by joining it to the 15-mer released from the 3' end of oligomer 1 as a result of the cleavage demonstrated on that strand. The parallel fraction obtained from mock purification with the parental plasmid served as the negative control in these experiments (Fig. 9B, lanes 1 and 3). Note that the 31-mer in lane 1 serves as an approximate marker for the 31-mer product in lane 4, and the 16-mer in lane 3 serves as an approximate marker for the 16-mer produced in lane 2, although these oligomers have different sequences. The activity demonstrated here was dependent upon the structure shown in Fig. 9A because heat denaturation of the complex before incubation with IN diminished the amount of product detected by over 70% (data not shown); residual susceptibility likely was due to some degree of reannealing to form complexes during the reaction. These results demonstrate that visna virus IN is able to catalyze in vitro the reverse of the integration reaction.

DISCUSSION

Visna virus, the prototypic lentivirus, causes degenerative neurologic disease in sheep (36), and many aspects of its biology suggest that it may serve as a useful model system for HIV and AIDS (38). Integration, considered a critical step in the retrovirus life cycle and a prime target for development of antiviral agents, has become amenable to study because of the development of oligonucleotide-based in vitro integrase assays

(27). We decided to purify and characterize the integrase from visna virus for two reasons. First, it has been reported that visna virus can productively infect sheep choroid plexus cells without detectable integration (18). Although this result may reflect the sensitivity of the methodology used to detect integration (i.e., Southern blotting), visna virus infection also has been associated with persistent high levels of unintegrated DNA (19). Thus, the formal possibility existed that visna virus IN is defective in enzymatic activities necessary to catalyze integration. A second impetus for studying visna virus IN was to create tools for comparative experiments with HIV-1 IN. Visna virus IN is particularly suited for such studies because a previous alignment and phylogenetic analysis had indicated that the integrases of HIV-1 and visna virus have separated from those of other retroviruses analyzed (44).

We found experimentally that purified visna virus IN appears to be a fully functional retroviral integrase. Similar to other integrases (9, 27, 42, 52), visna virus IN acts as a site-specific endonuclease to remove a dinucleotide from the 3' end of oligonucleotide substrates representing the termini of linear viral DNA (processing activity) and joins the recessed CA-3' OH ends of such DNA to other DNA sequences (integration activity). Optimal conditions for these two activities were found to be similar. In particular, there was an absolute requirement for Mn^{2+} as the divalent cation; visna virus IN was not active with Mg^{2+} , Ca^{2+} , or Zn^{2+} . A similar strong preference for Mn^{2+} over Mg^{2+} has been noted for MLV IN (9, 22) and human foamy virus IN (37). In contrast, integrases from AMV (15, 25, 27, 53), RSV (25, 28), HIV-1 (11, 49), and HIV-2 (52) do exhibit activity with Mg^{2+} , and one group reported that HIV-1 IN was active with Ca^{2+} (11), although another group could not detect such activity (49).

In general, despite the similarity in properties of different retroviral integrases that have been studied, the experimental conditions that support optimal activity for these proteins have varied greatly. For example, visna virus IN and MLV IN are progressively inhibited by 50 to 150 mM NaCl (Table 1) and 25 to 100 mM NaCl (22), respectively, but AMV IN has maximal activity at 145 mM NaCl (53) and HIV IN is fully active at 100 mM but inactive at 150 mM NaCl (11). Although we noted that visna virus IN did not require reducing agents, MLV IN apparently requires different types and amounts of reducing agents for its various activities (22). These diverse findings may indicate significant differences between proteins or may reflect the different purification protocols used and their effects on protein folding. Moreover, it is unclear how to extrapolate these observations to the microenvironment of the viral nucleoprotein complex within an infected cell (2). In vitro activities also may be affected by the ratio of protein to DNA used by various investigators. The ratio of 40 pmol of IN to 0.5 pmol of DNA used in the experiments reported here, although not kinetically optimal in enzymatic terms, may be of the same order of magnitude as the natural situation in which there are ~50 to 100 IN molecules per virion and two ends of linear DNA to be integrated in the infected cell (21).

Purified visna IN maintains a high degree of selectivity for cleavage after the CA that is two nucleotides from the ends of viral DNA. Although some activity was detected on apparently irrelevant oligonucleotides (Fig. 8), such terminal sequences would not be present in vivo. Indeed, the selectivity of integrases in vitro is remarkable, considering that recognition of the CA dinucleotides near viral DNA ends may be the only specificity required in vivo. In contrast, there is markedly diminished specificity for host DNA cleavage sites when the processed viral DNA termini act as the nucleophiles during strand transfer. Current models for retroviral integrases sug-

gest that all polynucleotidyl transfer reactions are catalyzed by a single active site (6, 13, 14, 24, 47, 48, 50, 52). How IN distinguishes whether viral or host DNA is the target for nucleophilic attack remains to be determined, but one possibility is that IN recognizes viral DNA and host DNA in different binding sites. It also is not clear how viral DNA can act sequentially as target (for processing) and then as nucleophile (for integration).

Although purified visna virus IN cleaved U5 and U3 substrates to comparable levels, there was a consistent suggestion that U3 substrates yielded more integration products. For AMV IN, the relevant U3 substrate has been reported to be more susceptible than the U5 substrate both for cleavage and integration (15, 53). In contrast, HIV-1 IN has usually (5, 11, 31, 33, 41, 46), but not always (51), appeared to have greater activity on HIV-1 U5 versus U3 sequences. Similarly, human foamy virus IN appeared to nick the cognate U5 substrates more efficiently than U3 sequences (37). The different results obtained for previously described integrases can be explained by dual biologic roles for the less susceptible DNA end that prevented that sequence from evolving solely to maximize susceptibility for integration. For example, HIV-1 U3 sequences also are part of the *nef* gene (5), human foamy virus U3 sequences code for part of *bel3* (37), and RSV U5 sequences form a secondary structure in RNA that has been shown to be important for initiation of reverse transcription (8). We cannot predict the preference for visna DNA ends in this way, because visna virus U3 sequences form the end of the *env* gene (44) and visna virus U5 sequences also have the potential to form a secondary structure (see below), although a biologic role for this structure has not been demonstrated. Furthermore, care must be taken in reaching conclusions based on these in vitro assays, as the contribution of the quality of the different oligonucleotide preparations, both labeled and unlabeled, is unknown. Whether integration of two viral DNA ends in vivo occurs in a concerted fashion, or sequentially with or without preference for one end, remains to be determined. It is interesting, however, that a mutation at one end of MLV DNA was found to result in diminished processing in vivo for both ends of linear DNA, suggesting some degree of cooperation between viral DNA ends during the process of integration (35).

We also examined a possible structural requirement for DNA substrates by testing the ability of visna virus IN to act on single oligonucleotides. We found that visna virus IN could cleave, but not integrate, visna virus U5 and U3 oligomers when presented without their complementary sequences (Fig. 7A and B). For AMV IN, the susceptibility of single RSV U5 positive strands was proved to be related to the potential to form intramolecular or intermolecular base pairs because of a short inverted repeat. Base substitutions that selectively disrupted the potential for base pairing were shown to markedly diminish susceptibility, whereas an oligonucleotide with a double substitution that maintained the potential base pairing, albeit with a different sequence, remained susceptible to cleavage (27). Consistent with those findings, the visna virus U5 positive strand has the potential to base pair intermolecularly at 10 of 11 positions (5'-CGGAGCGGATCTCGCAGC3', where underlined bases can pair between two identical molecules and the CA is in boldface), and the visna virus U3 negative strand has the potential to base pair intermolecularly at 10 of 12 positions (5'-GTTCTCTGTCCTGACAGT3').

Although the A of the CA dinucleotide would not be base paired in the U5 positive-strand potential structure described above, a similar finding was noted for the RSV U5 sequence (27). Moreover, it has been shown that HIV-1 substrates in

which the terminal three nucleotides were not base paired did get cleaved efficiently by HIV-1 IN, although such substrates were not integrated (41), consistent with our observations. In contrast to these results, oligonucleotides derived from the termini of HIV-1, although able to be specifically cleaved by visna virus IN when presented as duplex substrates with their complementary strands, could not be specifically cleaved when presented alone (Fig. 7C and D). Neither of these sequences has the potential for base pairing comparable to that possible for visna virus terminal sequences (17). Thus, the apparent variability in activity of different retroviral integrases on ostensibly single-stranded substrates may be an artifact due to secondary structures formed by some DNA sequences. It is likely that all retroviral integrases have an absolute requirement for double-stranded viral DNA in the processing and integration reactions. However, the same is not true for the disintegration reaction, in which a single nucleotide can replace the viral duplex surrogate in the disintegration substrate (7) (see below).

Visna virus IN also exhibited the ability to reverse the integration reaction (Fig. 9). This process requires both an endonucleolytic cut after the conserved CA dinucleotide, although this time the CA is internal to the DNA and not followed by authentic viral DNA bases, as well as ligation of two stretches of nonviral DNA. It is possible that IN catalyzes these two effects via a one-step transesterification reaction with the juxtaposed 3' OH of the nonviral DNA as the nucleophile, utilizing the same enzymatic site as for the forward reaction (6, 13, 14, 24, 47, 48, 50, 52). The biological function of this activity of IN is not known, but roles in retroviral recombination (7) and in the final repair steps of integration (i.e., removal of the two nucleotide overhang at the 5' end of viral DNA and ligation of this 5' phosphate end to a 3' OH group of host DNA [7, 41]) have been suggested. Of the endonuclease, integration, and disintegration activities of IN, the last appears to be the least constrained by sequence requirements, since some disintegration activity has been demonstrated for HIV-1 IN when the duplex viral sequences are replaced by only one (7) or two (41) nucleotides, which need not even be of viral origin (41). Similarly, reaction conditions seem least stringent for disintegration assays, since MLV IN was active for disintegration under conditions of pH and salt concentration which did not support processing activity (22). For these reasons, we did not explore this assay further. However, demonstration that visna virus IN can catalyze reversal of the integration reaction suggests that this protein, like other retroviral integrases, functions as an enzyme and is not consumed by the integration pathway (7).

In summary, we have demonstrated that purified visna virus IN exhibits the full repertoire of *in vitro* integrase functions. Although *in vivo* constraints may affect enzymatic activity, it is unlikely that high amounts of unintegrated visna virus DNA found experimentally reflect the function or quality of visna virus IN. Moreover, the phylogenetic relatedness of HIV and visna virus integrases, coupled with the subtle differences that have been identified between these enzymes, will permit comparative analyses that may shed further light on domains of this critical retroviral protein (6, 10–13, 23, 24, 29, 30, 32, 34, 40, 47–50, 54).

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