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# Catalytic Activities of the Human T-Cell Leukemia Virus Type II Integrase



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Despite the widespread nature of HTLV-II in New World populations and intravenous drug users, the enzymatic activities of the *pol* genes have not been reported. To ascertain the activity of the HTLV-$II_{G12}$ integrase (IN), the coding region was isolated and the encoded protein was purified, using nickel-affinity chromatography, to greater than 90% homogeneity. HTLV-$II_{G12}$ IN proved active on HTLV-$II_{G12}$ and HIV-1 integration and disintegration substrates. Distinct differences in requirements for enzyme concentration for 3′-processing, strand-transfer, and disintegration reactions were observed. Catalysis of integration reactions occurred in the presence of either $Mn^{2+}$ or $Mg^{2+}$, although strand-transfer activity preferred $Mn^{2+}$. In comparison, HTLV-$II_{G12}$ IN catalyzed disintegration reactions with almost 10-fold less protein, was not selective for $Mn^{2+}$ or $Mg^{2+}$, and tolerated higher NaCl concentrations than integration. HTLV-$II_{G12}$ IN was unable to catalyze the "splicing" reaction, which suggests that this may not be an activity ubiquitous to all retroviral integrases. 

## INTRODUCTION

Human T-cell leukemia virus type $II_{G12}$ (HTLV-$II_{G12}$)[3] was originally isolated from an asymptomatic Guaymi Indian in Panama (Pardi *et al.,* 1993). This population shows an 8 to 9% seropositive reaction for HTLV. In contrast to human T-cell leukemia virus type I, little is known about the natural history of infection, pathogenicity, and encoded enzyme activities of HTLV-II. Originally isolated from a patient with an atypical hairy T-cell leukemia (Chen *et al.,* 1983; Kalyanaraman *et al.,* 1982), subsequent screening found HTLV-II endemic to fairly "closed" societies such as the Amerindian populations of Florida (Levine *et al.,* 1993) and New Mexico (Hjelle *et al.,* 1991) and the native Indians of Panama (Pardi *et al.,* 1993). In studies of the Navajo and Pueblo Indians of New Mexico, HTLV-II was not correlated with an increased risk for hairy cell leukemia, chronic leukemia, or mycosis fungoides in a population with a fairly high rate of HTLV-II infection (1.0 to 1.6%). A significantly increasing number of HIV-1-seropositive intravenous drug abusers (IVDAs) are coinfected with HTLV-II (de-The and Bomford, 1993; Hall *et al.,* 1992; Lee *et al.,* 1989). In these coinfected populations, the presence of HTLV-II may affect progression of HIV-1 disease (Kaplan *et al.,* 1991; Page *et al.,* 1990; Wang *et al.,* 1993).

Integration provides an essential step in the retroviral life cycle by inserting a DNA copy of the viral genome into the host genomic DNA (Brown, 1990; Grandgenett and Mumm, 1990; Varmus and Swanstrom, 1984). This event comprises two enzymatic steps, 3′-processing and strand transfer, mediated by the viral-encoded enzyme integrase (IN). Equally essential for this process are the long terminal repeat sequences (LTR) that flank the two ends of the linear reverse-transcribed retroviral DNA. 3′-Processing removes nucleotides from each 3′ end of the viral LTR, leaving the conserved CA recessed at the 3′ end (Brown *et al.,* 1989; Katzman *et al.,* 1989; Roth *et al.,* 1989). Strand transfer covalently joins the 3′ recessed end of each processed LTR to the target DNA by a one-step transesterification reaction (Engelman *et al.,* 1991). In the presence of a substrate that mimics an integration intermediate, IN mediates a reverse reaction of integration, disintegration (Chow *et al.,* 1992). All of these catalytic activities are readily reconstituted *in vitro* using DNA substrates and require only IN and the substrate in the presence of a metal ion (Craigie *et al.,* 1990; Katzman *et al.,* 1989).

This paper presents the first report of the purification and the associated integration and disintegration activities of HTLV-$II_{G12}$ IN. Yield and stability of the HTLV-$II_{G12}$ IN were excellent, and HTLV-$II_{G12}$ IN showed efficient integration activity in the presence of either $Mn^{2+}$ or $Mg^{2+}$. One enzymatic activity, "splicing," observed with other integrases such as HIV-1 and M-MuLV, was lacking. The properties of several INs are known, yet limited information exists on the specific activity for each reaction. We present results that show the optimum concentrations of HTLV-$II_{G12}$ IN enzyme required for 3′-pro-

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[3] Abbreviations used: IN, integrase; HTLV-II, human T-cell leukemia virus type II; HIV-1, human immunodeficiency virus type 1; M-MuLV, Moloney murine leukemia virus; IVDAs, intravenous drug abusers; nt, nucleotide(s); AMV, avian myeloblastosis virus.



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cessing, strand transfer, and disintegration activities differ significantly.

## MATERIALS AND METHODS

### Materials

Oligonucleotide primers were purchased from Anagen Technologies (Palo Alto, CA), restriction enzymes from Gibco BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA), Vent polymerase from New England Biolabs, $T_4$ DNA polynucleotide kinase from Gibco BRL, and crude [$\gamma$-$^{32}$P]ATP from ICN (Irvine, CA). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

### Cloning of the HTLV-II$_{G12}$ integrase gene

The full-length HTLV-II$_{G12}$ genome was a gift from Dr. Diane Pardi (Center for Disease Control, Atlanta, GA) (Pardi *et al.,* 1993). The coding region for HTLV-II$_{G12}$ IN was amplified by polymerase chain reaction (PCR) using Vent DNA polymerase starting at position 4290 at the 5′ end of the proviral genome. The primers, P14227-1193 (5′ AAGCTTCATATGCTTATCCCCCTGACGCCCC) and P20283-0294 (5′ CTCGAGCCCATGGTGTTGG), introduced *Nde*I and *Xho*I restriction sites (underlined) at the 5′ and 3′ ends, respectively, of the encoded HTLV-II$_{G12}$ IN DNA sequence. The 920-bp PCR-amplified DNA was digested with *Nde*I and *Xho*I, gel isolated, and glassfines purified (Vogelstein and Gillespie, 1979). The isolated gene was cloned into the *Nde*I–*Xho*I restricted enzyme sites of pET22B (Novagen, Madison, WI) to generate the HTLV-II$_{G12}$ IN expression vector pET22INHIIg. Ligation of the IN gene into the *Xho*I site adds the DNA sequence encoding a hexahistidine tag to the 3′ end. Expression of the HTLV-II$_{G12}$ IN protein is directly under the control of the T7/lac promoter, thus facilitating protein expression through addition of isopropyl-1-thio-$\beta$-D-galactopyranoside (IPTG) to the bacterial culture (Studier and Moffat, 1986).

### Protein expression and purification

HTLV-II$_{G12}$ IN protein was purified from pET22INHIIg-transformed *Escherichia coli* BL21(DE3) (Novagen) by a modification of the protocol described by Jonsson *et al.* (1993). Briefly, BL21(DE3) clones harboring the pETINHIIg construct were cultured in 1 liter of 2× YT media containing 200 $\mu$g of ampicillin per milliliter. Protein expression was induced by adding IPTG to a final concentration of 0.4 m*M* during early log phase (0.6 $A_{600}$ units). Cells were harvested after 2.5 hr of further incubation and solubilized in 40 ml of Buffer A [6 *M* guanidine HCl, 0.5 *M* NaCl, 20 m*M* Tris, pH 8.0, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.4% Nonidet-P40 (NP-40)] with mild agitation at room temperature for 1 hr. The insoluble fraction was removed by centrifuging the suspension for 30 min at 16,000 *g.* Solubilized protein from the clear supernatant was purified on a 1-ml nickel nitrilotriacetate agarose affinity column facilitated by the hexahistidine tag ($Ni^{2+}$-NTA; Qiagen, Chatsworth, CA). The eluted IN protein was collected in 10 1-ml fractions and samples were quantitated by Bradford's assay (Bradford, 1976) using Bio-Rad Reagent (Madison, WI). Protein elution was characterized by a tailing off from the column.

Column fractions were pooled and adjusted with Buffer A to five different concentrations of protein: 0.8, 2.5, 5.0, 10, and 16 $\mu$g/$\mu$l. Fractions were placed in 1 liter of 3 *M* urea in Buffer B [1 *M* NaCl, 40 m*M* HEPES, pH 7.4, 2 m*M* EDTA, 10 m*M* $\beta$-mercaptoethanol (BME), 0.4% NP-40, 10% glycerol]. The protein fractions were refolded by dialysis through a step-wise dilution of urea from 3 to 0 *M* in Buffer B. The denaturant was diluted with fresh Buffer B by 1 *M* urea every 24 hr over a 3-day period. A final dialysis of the fractions was conducted in Buffer C [200 m*M* NaCl, 40 m*M* HEPES, pH 7.4, 0.1 m*M* EDTA, 1 m*M* DTT, 0.4% NP-40, 10% glycerol] for a further 36 hr, which included one change of the buffer after 24 hr. In the final dialysis step, a protein precipitate was formed which was greater than 95% IN. The precipitated protein was centrifuged at 12,000 *g* for 5 min and resuspended in the final dialysis Buffer D [40 m*M* HEPES, pH 7.4, 500 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, 0.4% NP-40, 10% glycerol], which completely resuspended the protein precipitate. The fractions were frozen in dry ice and stored at −80°.

### SDS–PAGE, polyclonal antibody production, and Western analysis

Fifteen percent SDS–polyacrylamide gel electrophoresis was used to analyze column fractions and confirm protein size. Polyclonal antibodies to the HTLV-II$_{G12}$ IN were generated by injecting the highly purified protein into rabbits. TiterMax (Vaxel Inc., Norcross, GA) was used as adjuvant in a 1:1 ratio with the antigen (used as per directions of manufacturer). One milliliter of the purified and active HTLV-II$_{G12}$ IN protein (1 mg/ml) was combined with 1.0 ml of the TiterMax adjuvant and given as four 40-$\mu$l subcutaneous injections. After 14 days, 10 ml of the rabbit blood was collected and centrifuged, and the sera were frozen at −80°. Reactivity of the antibody was ascertained by Western analysis as described earlier (Jonsson *et al.,* 1993). HIV-1 IN was expressed from pJL10 in BL21(DE3) cells similar to the method described for HTLV-II$_{G12}$ IN (NIH Research and Reference Reagent Program).

### Integration and disintegration assays

Enzymatic activities of the purified HTLV-II$_{G12}$ IN, namely, 3′-processing, strand transfer, and disintegration were assayed using identical buffer conditions with standard *in vitro* radioactive-based assays (Jonsson *et al.,* 1993). Oligonucleotide sequences corresponding to the

terminal 20 bp from U5 and U3 LTRs of the HTLV-$II_{G12}$ viral DNA were used as substrates (Fig. 2B). All assays were performed in a 15-$\mu$l final reaction volume containing 25 m*M* MOPS (pH 7.2), 10 m*M* BME, 10% glycerol, 0.75 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 7.5 m*M* $MnCl_2$, 0.1 $\mu M$ $^{32}$P-labeled substrate, and 0.35 $\mu M$ integrase, unless otherwise specified in the figure legends. A 6.7 m*M* NaCl concentration is introduced into the assays due to salt carryover from the IN storage buffer (Buffer D). Reactions were incubated at 37° for 60 min and stopped by a further 30-min incubation with 25 $\mu$g of proteinase K per reaction in the presence of 25 m*M* EDTA and 0.01% SDS at 37°. Ten microliters of loading dye (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.5% xylene cyanol) was added following proteinase K digestion. Reactions were heated from 3 to 5 min at 95°, loaded, separated on 20% denaturing polyacrylamide gel, and subjected to autoradiography as previously described (Jonsson *et al.,* 1993).

### Substrate preparation

Oligonucleotides were purified on 20% denaturing polyacrylamide gels, $^{32}$P-labeled at the 5′ end with T4 polynucleotide kinase, and hybridized to complementary oligonucleotide strands as previously described (Jonsson *et al.,* 1993). Unincorporated radioactivity was removed from labeled integration and disintegration substrates with G-25 or G-50 Quick Spin columns (Boehringer Mannheim, IN).

## RESULTS

### Expression and purification of the HTLV-$II_{G12}$ IN protein

BL21(DE3) cells harboring the expression vector pET22HIIg were induced with IPTG to express the HTLV-$II_{G12}$ IN. Following the induction period, total protein was extracted under denaturing conditions. Solubilized protein was subsequently subjected to separation by nickel-affinity chromatography. Following removal of the majority of the contaminating proteins, HTLV-$II_{G12}$ IN was eluted from the resin by lowering the buffer pH to 4.5. This released HTLV-$II_{G12}$ IN in high concentrations into eight fractions representing 8 column volumes (Fig. 1A, lanes 4 to 11). As evident from Fig. 1A, high levels of HTLV-$II_{G12}$ IN expression were obtained in *E. coli* and the protein was readily extracted. Binding conditions were evidently efficient, as seen from the very low traces of IN in the column flowthrough (Fig. 1A, lane 12). This one-step affinity purification yielded approximately 70 mg of 90–95% homogenous HTLV-$II_{G12}$ IN from 1 liter of cells.

Column fractions were pooled and diluted to ascertain the optimal concentration for refolding as measured by the activity of the HTLV-$II_{G12}$ IN enzyme. Each of the refolded fractions was assayed for integration and disintegration activities (data not shown). The protein fraction refolded at a concentration of 5 mg/ml exhibited greatest enzymatic activity. Refolding at lower concentrations retained partial activity, while fractions refolded at higher protein concentrations had no activity. A slight increase in the purity of the protein was made following refolding by including a precipitation step. HTLV-$II_{G12}$ IN precipitated upon addition of Buffer C (0.2 *M* NaCl). The precipitated protein was resuspended in Buffer D (0.5 *M* NaCl) to a final concentration of 1 mg/ml. Greater than 95% of the precipitate was HTLV-$II_{G12}$ IN, which was highly stable and soluble in this high-salt environment.

The purified and active HTLV-$II_{G12}$ IN fraction refolded at a concentration of 5 mg/ml was chosen as antigen for the production of polyclonal antibodies in rabbits. Western analysis of crude total cell extract (Fig. 1B, lanes 1) and purified refolded HTLV-$II_{G12}$ IN (Fig. 1B, lanes 2) was examined for reactivity against sera from HTLV-$II_{G12}$ IN-inoculated rabbits. Reactivity of the appropriate-size protein, 32 kDa, was observed in crude and purified fractions (Fig. 1B, lanes 1 and 2). No cross-reactivity was noted against HIV-1 IN (Fig. 1B, lane 3). Further, no cross-reactivity with higher or lower protein bands occurred in the immunoanalysis. This suggests the expressed IN protein was stable and not affected by endogenous bacterial proteases.

### *In vitro* enzymatic activities of HTLV-$II_{G12}$ IN

HTLV-$II_{G12}$ IN was assayed for 3′-processing, strand transfer, and disintegration activities with LTR-specific substrates. The assay for 3′-processing uses a blunt-ended 20-bp DNA substrate that mimics the U5 end of the HTLV-$II_{G12}$ LTR (Fig. 2A, Step 1). Strand-transfer activity is detected by the use of a precleaved duplex DNA substrate (Fig. 2A, Step 2). The blunt-LTR substrate will also serve as a strand-transfer substrate following 3′-processing. HTLV-$II_{G12}$ IN also catalyzes the disintegration of a Y-shaped substrate that resembles an integration intermediate (Fig. 2A, Step 3). Oligonucleotides used in the synthesis of the substrates in these experiments are listed in Fig. 2B.

3′-Processing, strand transfer, and disintegration were measured over a 10-fold range of protein concentration under identical reaction conditions (Fig. 3A). Using the blunt-end substrate, 3′-processing and strand-transfer activities of the HTLV-$II_{G12}$ IN were clearly visible at a protein concentration of 0.35 pmol/$\mu$l (Fig. 3A, lane 7). This concentration represents a 5:1 ratio of protein to substrate. At a 2-fold lower concentration of protein (0.17 pmol/$\mu$l), 3′-processing activity was only faintly detectable (Fig. 3A, lane 6), and strand-transfer products were not observed. Further dilution in protein concentration did not reveal any processed products even with longer exposures to X-ray film (Fig. 3A, lanes 2–5). Thus, with respect to 3′-processing activity, only the higher range

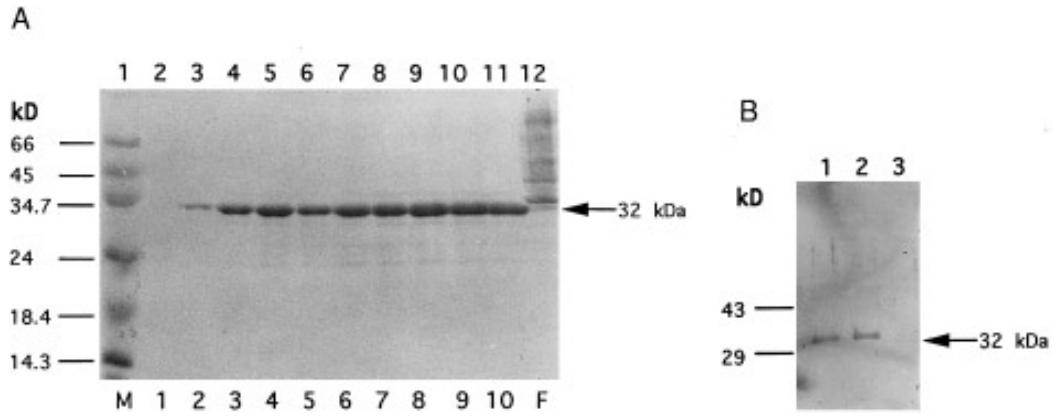


FIG. 1. Purification of HTLV-II$_{G12}$ IN. (A) HTLV-II$_{G12}$ IN protein fractions eluted from the $Ni^{2+}$ NTA column were separated by 15% SDS–PAGE and visualized with Coomassie blue staining. Lane numbers are indicated at the top and column fractions are indicated at the bottom. Lanes 2 through 11 represent column fractions 1 to 10 from the pH 4.5 elution step. The major band indicated by the arrow in lanes 3 to 11 corresponds to the HTLV-II$_{G12}$ IN protein of approximately 32 kDa. Lane 12 shows the flowthrough from the column after loading. Lane 1 contains molecular weight markers (M). (B) Immunoassay of HTLV-II$_{G12}$ IN purified from *E. coli* transformed cells carrying the expression vector pET22HIIg is shown. Crude HTLV-II$_{G12}$ IN (lane 1), refolded HTLV-II$_{G12}$ IN (lane 2), and crude HIV-1 IN (lane 3) were separated by 15% SDS–PAGE, transferred on to Nytran membrane, and incubated with a 1 to 500 dilution of rabbit antisera raised against HTLV-II$_{G12}$ IN as described under Materials and Methods. The positions of molecular weight markers are indicated to the left.

of protein concentration examined produced products. HTLV-II$_{G12}$ IN also showed dinucleotide cleavage activity with the HTLV-II$_{G12}$ U3 LTR; however, this activity was notably less than that observed for the U5 LTR substrate (M. Balakrishnan and C. B. Jonsson, data not shown). With precleaved substrate, HTLV-II$_{G12}$ IN catalyzed strand-transfer reactions at IN concentration of 0.17 and 0.35 pmol/$\mu$l (Fig. 3A, lanes 13–14). Longer exposures

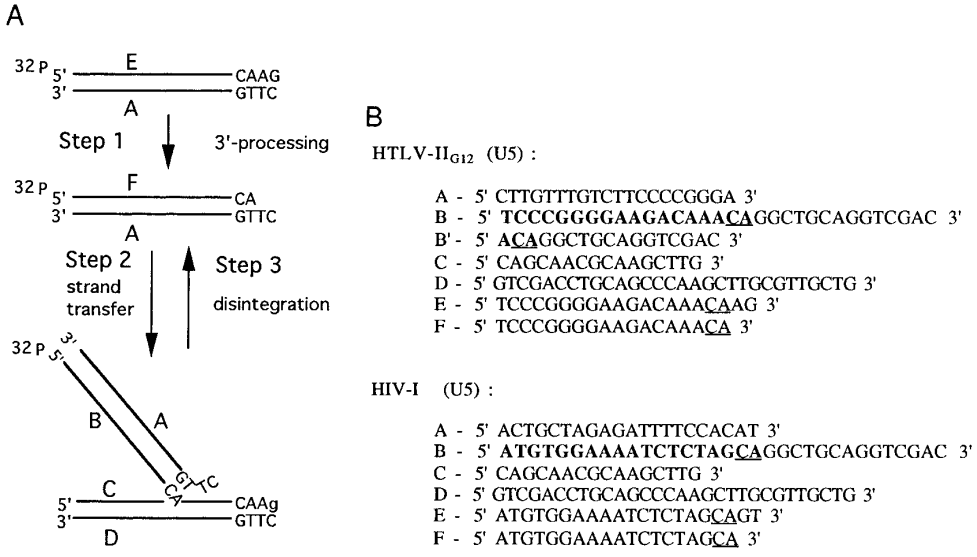


FIG. 2. IN LTR assays and oligonucleotide substrates. (A) Schematic representation of the enzymatic activities mediated by HTLV-II$_{G12}$ IN, 3′-processing (Step 1), strand transfer (Step 2), and disintegration (Step 3) are shown. Integration substrates consist of two complementary 20-nucleotide oligomers that mimic the terminal U5 or U3 end of the viral LTR (see Fig. 2B). Strand E, which undergoes 3′-processing at the 3′ end is $^{32}$P labeled at its 5′ end (Step 1). The 3′-processing activity is thus detected by the generation of the 18-nt recessed product. During strand transfer, the processed LTR serves as a substrate for a one-step transesterification reaction mediated by the HTLV-II$_{G12}$ IN (Step 2). Strand transfer into the target DNA is random, thus generating a spectrum of larger and smaller sized products. The disintegration substrate represents a hypothetical strand-transfer intermediate (Step 3). This reaction is followed by $^{32}$P labeling the 5′ end of the C strand. Joining of the 3′ end of the C strand to the B strand results in the formation of a 30-nt product. (B) Oligonucleotide sequences derived from the U5 termini of HTLV-II$_{G12}$ and HIV-1 and used in the synthesis of the substrates are shown. The substrates used to follow 3′-processing activity were synthesized by hybridizing together the E and A strands. The substrate used to measure strand-transfer activity contained the F and A. The disintegration substrates consisted of the A, B, C, and D strands. For the A- overhang disintegration substrate the B′ strand was used in place of the B strand, while the single-stranded disintegration substrate lacked the A strand. The conserved -CA residues in the positive strands are underlined. Sequences representing the LTR region in the B strands are shown in bold.

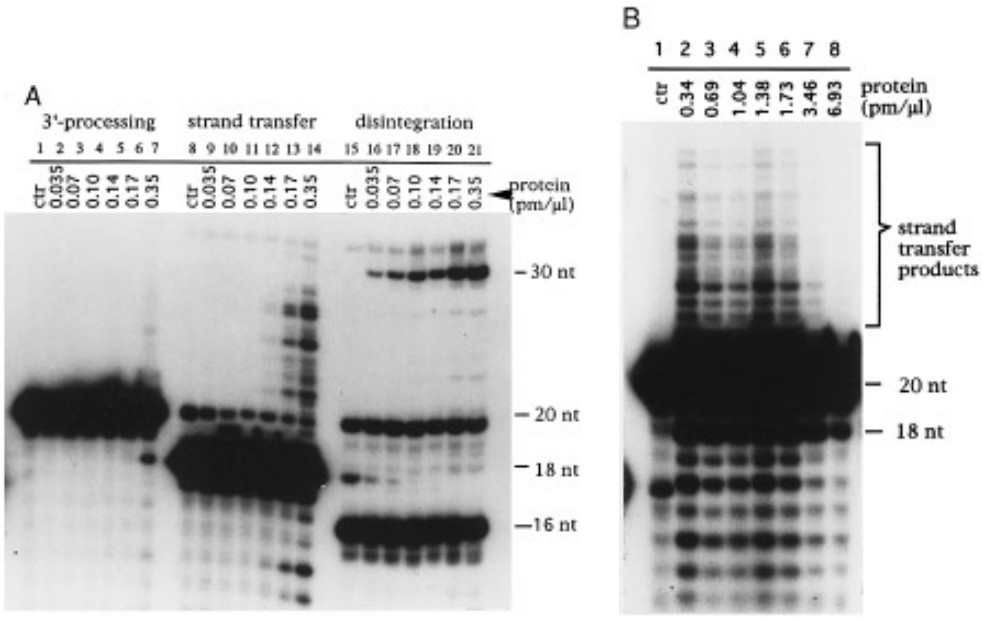


FIG. 3. Enzymatic activities of HTLV-II$_{G12}$ IN as a function of protein concentration. (A) HTLV-II$_{G12}$ IN enzymatic activities were assayed as described under Materials and Methods using HTLV-II U5 LTR-derived substrates. Enzymatic activity is shown in reactions with increasing concentration of protein for 3′-processing, lanes 1–7; strand transfer, lanes 8–14; and disintegration, lanes 15–21. The protein concentrations (pmol/$\mu$l) are indicated directly above each lane. Assays were done in 15-$\mu$l reaction volumes with a final substrate concentration of 0.07 pmol/$\mu$l. Reactions of substrate without protein are shown in the first lane of each reaction set. The 18-nucleotide species in lanes 15–21 is due to formation of secondary structures during substrate synthesis and has been observed before (29). (B) 3′-Processing and strand-transfer activities using increasing concentrations of HTLV-II$_{G12}$ IN were examined with the blunt-ended HTLV-II U5 LTR substrate. Enzyme activity in presence of increasing concentrations of protein is shown in lanes 2–8. Protein concentrations are indicated directly above each lane. Substrate concentration used in these assays was 0.07 pmol/$\mu$l. Reaction with substrate in the absence of protein is represented in lane 1. In A and B, reactions were incubated at 37° for 60 min and terminated with proteinase K treatment for 30 min at 37°.

showed reactions containing 0.14 pmol/$\mu$l HTLV-II$_{G12}$ IN, active for strand transfer (Fig. 3A, lane 12). This represents a 2:1 ratio of substrate to protein concentration in the assay. No activity was observed in reactions containing 0.035–0.10 pmol/$\mu$l of IN (Fig. 3A, lanes 9–11). Strand-transfer activity required approximately 2-fold less protein compared to 3′-processing reaction.

In contrast to 3′-processing and strand-transfer assays, the disintegration reaction yielded a 30-nucleotide product at the lowest HTLV-II$_{G12}$ IN concentration examined, 0.035 pmol/$\mu$l (Fig. 3A, lane 16). This concentration of IN reflects a 1:2 ratio of protein to substrate in the assay. The level of activity increased with increasing protein concentration (Fig. 3A, lanes 16 to 21). Disintegration activity required approximately 10-fold less HTLV-II$_{G12}$ IN protein compared to the 3′-processing and 5-fold less compared to strand transfer. The IN concentrations needed to observe 3′-processing, strand transfer, and disintegration activities thus showed distinct variation.

In all enzymatic assays, HTLV-II$_{G12}$ IN activity was inhibited at protein concentrations above 2.0 pmol/$\mu$l (Fig. 3B, and data not shown). In Fig. 3B, the blunt-ended integration substrate serves to show both 3′-processing and strand-transfer activities at protein concentrations ranging from 0.34 pmol/$\mu$l (Fig. 3B, lane 2) to 6.93 pmol/$\mu$l (Fig. 3B, lane 8). 3′-Processing activity fell approximately twofold at 3.46 and 6.9 pmol/$\mu$l IN concentration (Fig. 3B, lanes 7 and 8). Strand-transfer activity decreased substantially as protein concentrations were increased above 1.7 pmol/$\mu$l (Fig. 3B, lanes 7 and 8). At 6.9 pmol/$\mu$l, strand-transfer products were undetectable. Strand-transfer activity with the 3′-processed substrate showed similar inhibitory effects at the higher IN concentrations of 3.46 and 6.9 pmol/$\mu$l (data not shown). A time course measured enzymatic activity for HTLV-II$_{G12}$ IN over a period of 60 min to compare the kinetics of 3′-processing, strand transfer, and disintegration (data not shown). With the blunt-LTR substrate, the 3′-processed product appeared within 2 min of incubation followed by strand-transfer products by the end of 5 min. Products from HTLV-II$_{G12}$ IN-catalyzed strand-transfer and disintegration reactions were observed in less than 1 min. As observed in protein to substrate titration, the time course highlights the kinetic efficiency of strand transfer as opposed to the relatively slower 3′-processing.

## Ionic strength, metal ion, and temperature requirements

The effect of increasing sodium chloride concentration and metal ion requirement was examined for the *in vitro* integration and disintegration reactions catalyzed by

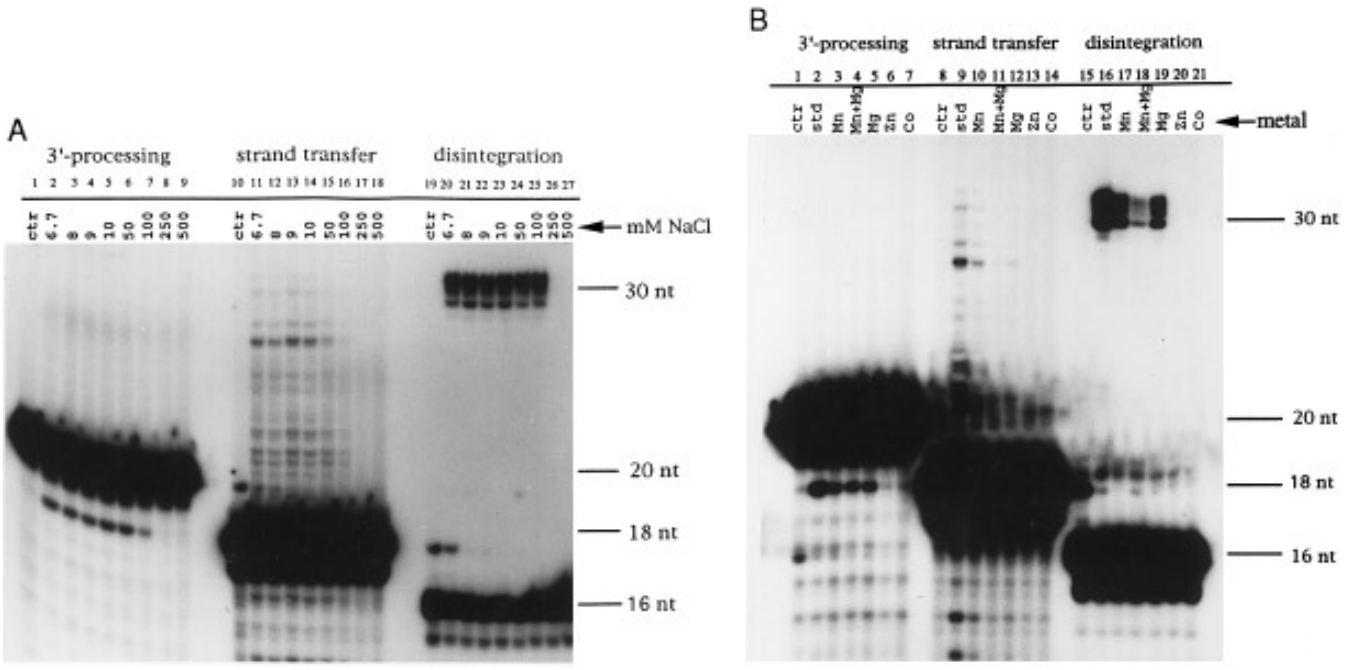


FIG. 4. Salt and metal requirements of HTLV-II$_{G12}$ IN enzymatic activities. (A) The optimal NaCl concentration for 3′-processing (lanes 1–9), strand-transfer (lanes 10–18), and disintegration (lanes 19–27) activities for HTLV-II$_{G12}$ IN were investigated. The NaCl concentrations are indicated directly above each lane. Reactions of substrate without protein are shown in the first lane of each reaction set (lanes 1, 10, and 19). Standard reaction conditions that contain 6.7 m*M* NaCl and which were used throughout the study are represented in the second lane of each reaction set (lanes 2, 11, and 20). Subsequent lanes show reactions conducted with increasing NaCl concentrations of 8 m*M* (3, 12, and 21), 9 m*M* (lanes 4, 13, and 22), 10 m*M* (lanes 5, 14, and 23), 50 m*M* (lanes 6, 15, and 24), 100 m*M* (lanes 7, 16, and 25), 250 m*M* (lanes 8, 17, and 26), and 500 m*M* (lanes 9, 18, and 27). (B) Metal requirements for optimal 3′-processing (lanes 1–7), strand-transfer (lanes 8–14), and disintegration (lanes 15–21) activities were examined for HTLV-II$_{G12}$ IN. Reactions containing radiolabeled substrate in the absence of protein are represented in the first lane of each reaction set (lanes 1, 8, and 15). Reactions done under standard buffer conditions with 7.5 m*M* $Mn^{2+}$ are represented in the second lane of each reaction set (lanes 2, 9, and 16). Lanes 3–7, 10–14, and 17–21 represent reactions where the 7.5 m*M* $Mn^{2+}$ was substituted with 10 m*M* $Mn^{2+}$ (lanes 3, 10, and 17), 5 m*M* $Mn^{2+}$ and 5 m*M* $Mg^{2+}$ (lanes 4, 11, and 18), 10 m*M* $Mg^{2+}$ (lanes 5, 12, and 19), 10 m*M* $Zn^{2+}$ (lanes 6, 13, and 20), and 10 m*M* $Co^{2+}$ (lanes 7, 14, and 21). In both A and B substrate for 3′-processing is represented by the 20-nt band and the 3′-processed product is detected as the shortened 18-nt band. The precleaved substrate used to detect strand-transfer activity is represented by the 18-nt band, while the strand-transfer products are detected by the presence of the slower migrating bands above the substrate bands. The disintegration substrate is represented by the 16-nt band, while the disintegration product is represented by the 30-nt band. Assays were performed in 15-$\mu$l reaction volume with 0.1 $\mu M$ labeled substrate and 0.35 $\mu M$ IN.

HTLV-II$_{G12}$ IN. Enzymatic activity was compared under identical conditions for each of the three substrates in the presence of increasing salt concentrations (Fig. 4A). The 6.7 m*M* NaCl at the lower end of the salt titration represents carryover from the IN storage buffer (see Materials and Methods). With the blunt-LTR substrate, 3′-processed product was detectable through 100 m*M* NaCl concentration (Fig. 4A, lanes 2–7). The level of 18-nucleotide product decreased at 100 m*M* NaCl (Fig. 4A, lane 7). Higher salt concentrations of 250 and 500 m*M* NaCl inhibited 3′-processing completely (Fig. 4A, lanes 8 and 9). The assay for strand-transfer activity tolerated salt concentrations through 50 m*M* (Fig. 4A, lanes 11–15). Strand-transfer activity was notably reduced at 100 m*M* NaCl concentrations (Fig. 4A, lane 16). At salt concentrations above 100 m*M,* strand-transfer products were absent (Fig. 4A, lanes 17 and 18). We observed equivalent levels of disintegration activity from 6 to 100 m*M* NaCl (Fig. 4A, lanes 19–25), but not at 250 and 500 m*M* NaCl (Fig. 4A, lanes 26–27). However, of the three HTLV-II$_{G12}$ IN activities, disintegration activity proved the most substantial in a higher salt environment.

Integration and disintegration activities of HTLV-II$_{G12}$ IN were examined in the presence of four different divalent cations, namely, $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$, and $Co^{2+}$ (Fig. 4B). Integration reactions of IN have an absolute requirement for magnesium or manganese. To date, disintegration activity has only been measured with $Mn^{2+}$. HTLV-II$_{G12}$ IN catalyzed both 3′-processing and strand-transfer activities in the presence of either 10 m*M* $MnCl_2$ (Fig. 4B, lanes 3 and 10) or 10 m*M* $MgCl_2$ (Fig. 4B, lanes 5 and 12), but no enzyme activity was observed in the presence of $ZnSO_4$ (Fig. 4B, lanes 6 and 13) or $CoCl_2$ (Fig. 4B, lanes 7 and 14). This closely compares with the standard buffer conditions used herein ($MnCl_2$ at 7.5 m*M,* Fig. 4B, lanes 2 and 9). While the cleavage step was catalyzed equally well in the presence of either of the cations (Fig. 4B, lanes 3 and 5), strand-transfer activity

showed a distinct preference for $Mn^{2+}$ (Fig. 4B, lanes 10 and 12). Disintegration activity showed a trend similar to 3′-processing, working equally well in $MnCl_2$ or $MgCl_2$ environment (Fig. 4B, lanes 17–19); however, no enzyme activity was observed in the presence of $ZnSO_4$ (Fig. 4B, lane 20) or $CoCl_2$ (Fig. 4B, lane 21). An interesting observation in these studies was the effect of mixing $MnCl_2$ and $MgCl_2$ in the HTLV-$II_{G12}$ IN reactions. 3′-Processing was catalyzed as efficiently in the presence of $MnCl_2$ or $MgCl_2$ or in their mixed presence (Fig. 4B, lanes 3–5), while strand transfer (Fig. 4B, lanes 10–12) and disintegration (Fig. 4B, lanes 17–19) were reduced. Finally, no significant difference in the final levels of reaction products were noted at 30 or 37° (M. Balakrishnan and C. B. Jonsson, data not shown).

### Catalysis with HIV-1 LTR substrates

IVDAs are quite often co-infected with both HTLV-II and HIV-1, and the viruses are capable of interaction *in vivo* (de-The and Bomford, 1993; Hall *et al.,* 1992; Lee *et al.,* 1989; Wang *et al.,* 1993). Whether these two retroviruses could integrate one another's genomes is unknown. To gain further insight, we measured HTLV-$II_{G12}$ IN activity for 3′-processing, strand transfer, and disintegration using HIV-1 U5 LTR integration and disintegration substrates.

HTLV-$II_{G12}$ IN showed efficient cleavage of the blunt U5 LTR substrate from HIV-1 (Fig. 5A, lane 4). Strand-transfer activity was also observed with the precleaved HIV U5 LTR substrates, though the levels of activity were lower than that observed with the HTLV-$II_{G12}$ LTR (compare Fig. 5A, lane 8 to lane 6). Interestingly, the pattern of strand-transfer activity observed with the HTLV-$II_{G12}$ IN on the HIV LTR appeared identical to that observed with the HIV-1 IN on its own LTR (M. Balakrishnan and C. B. Jonsson, unpublished results). The HTLV-$II_{G12}$ and HIV-1 disintegration substrates differ in sequences only in their LTR arms and are identical in the target. HTLV-$II_{G12}$ IN catalyzed disintegration reaction with the HIV-1 Y substrate, generating the 30-nt product (Fig. 5B, lane 4). Activity on the HTLV-$II_{G12}$ Y substrate is shown in Fig. 5B, lane 2. Preference for the HTLV-$II_{G12}$ substrates over the HIV-1 substrates by HTLV-$II_{G12}$ IN was clearly more pronounced with the integration substrates than with the disintegration substrates (compare Figs. 5A and 5B). Therefore, sequence played a more crucial role in substrate recognition for the integration activity of HTLV-$II_{G12}$ IN than for disintegration activity.

### Splicing activity of HTLV-$II_{G12}$ IN

The splicing activity of HIV-1 IN demonstrates a remarkable enzymatic flexibility marked by its ability to catalyze reactions with single-stranded disintegration substrates (single-stranded Y substrate, Fig. 6A) and with a single nucleotide overhang disintegration substrate (A-overhang Y substrate, Fig. 6A) (Chow *et al.,* 1992). In contrast, the splicing activity with the M-MuLV IN shows a strict requirement for the LTR portion of the disintegration substrate and only catalyzes reactions with at least 17 nucleotides of the LTR in a single-stranded form (Donzella *et al.,* 1993). The molecular basis for these differences is unknown. It was of interest to compare these activities with HTLV-$II_{G12}$ IN's activities to define which type of enzymatic activity it would have, and perhaps to lend insight into whether the HTLV-$II_{G12}$ IN was similar catalytically to HIV-1 IN or M-MuLV IN.

Two prototype single-stranded disintegration substrates were examined for HTLV-$II_{G12}$ IN activity (Fig. 6B). The standard Y substrate, the single-stranded, and the A- overhang Y substrates are identical in sequence in the C and D regions. The three disintegration substrates differ only in the viral LTR. Activity with the standard double-stranded disintegration substrate was measured for comparison (Fig. 6B, lane 2). The A- overhang Y substrate contained a shortened B strand (B′) having a single nucleotide instead of the LTR arm (Fig. 6A). Enzymatic activity of HTLV-$II_{G12}$ IN with the A- overhang Y substrate was not observed even with longer exposures in several attempts (Fig. 6B, lane 4). The single-stranded Y substrate contained 19 nucleotides of the HTLV-$II_{G12}$ LTR in the single-stranded form, while the remainder of the substrate was double-stranded (Fig. 6A). The 30-nucleotide disintegration product was not detected with the single-stranded Y substrate (Fig. 6B, lane 6). It was surprising that no activity was detected with either of these disintegration substrates. This suggests that the splicing activity may not be common to all retroviral integrases.

## DISCUSSION

HTLV-$II_{G12}$ IN expressed in and purified from *E. coli* was highly active, catalyzing integration and disintegration activities characteristic of this class of proteins. Unique to this characterization study were protein titrations that defined a critical concentration of HTLV-$II_{G12}$ IN required to bring about the different enzymatic activities, 3′-processing, strand transfer, and disintegration. We estimate that while 3′-processing required a 5:1 ratio of protein:substrate concentration in the assay, strand transfer, in the presence of the precleaved LTR, required only a 2:1 ratio to generate integration products. Thus, the initial step of LTR processing required approximately twice the amount of protein than was required to catalyze strand transfer. Disintegration was clearly the most rapidly catalyzed reaction requiring 10- and 5-fold less protein compared to the 3′-processing and strand-transfer activities of the HTLV-$II_{G12}$ IN, respectively.

Our studies show the HTLV-$II_{G12}$ IN to be fairly tolerant to and stable at moderate salt concentrations ($<$100 m*M* NaCl), and optimal activity could be attained at as low as 6.7 m*M* NaCl. This was comparable to HIV-1 IN which

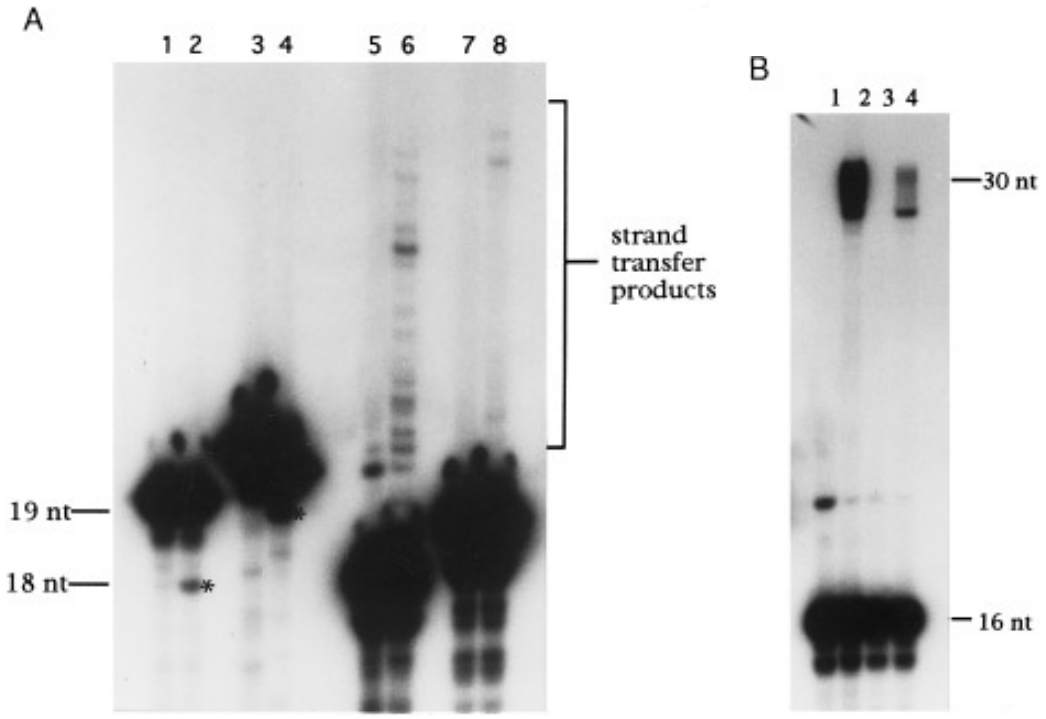


FIG. 5. Activity of HTLV-II$_{G12}$ IN with HIV-1 substrates. HTLV-II$_{G12}$ IN was assayed using HTLV-II$_{G12}$ and HIV-1 U5 LTR-derived substrates. (A) 3′-Processing (lanes 1–4) and strand-transfer (lanes 5–8) activities are presented for HTLV-II$_{G12}$ IN. 3′-Processing activities of HTLV-II$_{G12}$ IN with the HTLV-II LTR (lane 2, 18-nt product) and HIV-1 LTR (lane 4, 19-nt product) are shown. Products are indicated by asterisk. Substrate assays without the addition of IN are shown in lanes 1 and 3. Strand-transfer reactions (lanes 5–8) with HTLV-II$_{G12}$ LTR (lane 5 and 6) and HIV-1 LTR (lane 7 and 8) are shown for HTLV-II$_{G12}$ IN. Strand-transfer products are represented by the slower migrating bands (lanes 6 and 8). Substrate reactions without IN are shown for comparison in lanes 5 and 6. (B) Disintegration substrates (16 nt) from HTLV-II$_{G12}$ (lanes 1 and 2) and HIV-1 (lanes 3 and 4) are represented by the 16-nt bands. Even-numbered lanes show substrate reactions in the presence of HTLV-II$_{G12}$ IN protein. Disintegration products are indicated by the presence of a 30-nt band (lanes 2 and 4). Odd-numbered lanes show substrate reactions in the absence of HTLV-II$_{G12}$ IN protein. In both A and B assays were performed as described under Materials and Methods with 0.1 $\mu M$ labeled substrate and 0.35 $\mu M$ HTLV-II$_{G12}$ IN.

is fully active at 100 m*M* NaCl (Drelich *et al.,* 1992). Comparing other INs, AMV IN has a higher salt requirement, showing maximal activity at 145 m*M* NaCl (Vora *et al.,* 1990), while M-MuLV and visna virus INs are progressively inhibited by 25 to 100 m*M* NaCl (Jonsson *et al.,* 1993) and 50 to 150 m*M* NaCl (Katzman and Sudol, 1994), respectively. HTLV-II$_{G12}$ IN catalyzed 3′-processing and disintegration with equal efficiency in the presence of either $Mn^{2+}$ or $Mg^{2+}$. Strand-transfer activity was also mediated in presence of either of the divalent cations, although there was a definite preference for $Mn^{2+}$. Interestingly, adding both $Mn^{2+}$ and $Mg^{2+}$ in the reaction environment lowered strand-transfer and disintegration activities, but not 3′-processing. Studies on the AMV IN (Vora *et al.,* 1990; Fitzgerald *et al.,* 1992), ASLV IN (Katz *et al.,* 1990; Katzman *et al.,* 1991), HIV-1 IN (Vincent *et al.,* 1993; Engelman and Craigie, 1995), and HIV-2 IN (Vink *et al.,* 1991) have also reported various levels of integration activity with $Mg^{2+}$. In contrast, INs from M-MuLV (Craigie *et al.,* 1990; Jonsson *et al.,* 1993), FIV (Vink *et al.,* 1994), visna virus (Katzman and Sudol, 1994), and human foamy virus (Pahl and Fugel, 1993) exhibit strong preference for $Mn^{2+}$ with no detectable activity in the presence of $Mg^{2+}$. The role of manganese ion in controlling the proposed stereochemistry of the polynucleotidyl transfer during integration is not understood at present. Precedents from studies of phosphoryl and nucleotidyl transfer reactions would suggest that the manganese ion has an important role in acceleration of the $S_n2$ reaction pathway by binding the entering group or the phosphoryl group (Steitz and Steitz, 1993).

HTLV-II$_{G12}$ IN did not have a strict specificity for its own LTR, and it recognized blunt-ended and processed LTRs from HIV-1. Certain retroviral integrases have been reported to act on heterologous LTR substrates, although no obvious recognition sequences among these LTRs have been identified (Bushman and Craigie, 1990; Katzman *et al.,* 1989; Katzman and Sudol, 1994; Leavitt *et al.,* 1992; Sherman *et al.,* 1992). Our studies showed that HTLV-II$_{G12}$ IN also possessed this flexibility, recognizing both HTLV-II and HIV-1 U5 LTR substrates. Difference in sequence requirements for disintegration has also been observed for M-MuLV IN (Donzella *et al.,* 1993; Jonsson and Roth, 1993). Of interest, HTLV-II$_{G12}$ IN did not catalyze reactions with the disintegration "splicing" substrates. To date, only HIV-1 IN demonstrates this enzymatic activity

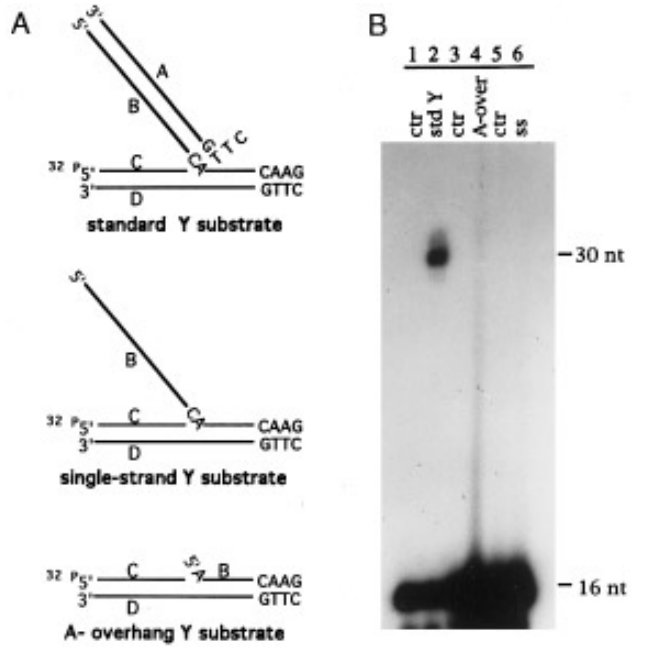


FIG. 6. Disintegration reactions catalyzed by HTLV-II$_{G12}$ IN using standard and modified disintegration substrates. (A) Schematic representation of the three different disintegration substrates used in these assays. The standard Y substrate is represented in the uppermost schematic. The single-stranded Y substrate (center) was generated by omission of the A strand during hybridization. The A- overhang Y substrate (bottom) was synthesized by omitting the A strand and using the shortened B′ strand. (B) Enzymatic assays using the standard Y substrate (lanes 1 and 2), A- overhang Y substrate (lanes 3 and 4), and the single-stranded Y substrate (lanes 5 and 6) are shown for HTLV-II$_{G12}$ IN. Even-numbered lanes show reactions in the presence of HTLV-II$_{G12}$ IN protein. Odd-numbered lanes show reactions in the absence of HTLV-II$_{G12}$ IN protein. Substrates are represented by the 16-nt band and products are detected by the presence of the 30-nt band. Disintegration reactions were performed under identical conditions with each substrate as described under Materials and Methods in the presence of 0.1 $\mu M$ substrate and 0.35 $\mu M$ IN.

on a single A- overhang disintegration substrate (Chow *et al.,* 1992). M-MuLV IN requires 17 nucleotides in the single-stranded region to catalyze this splicing activity (Donzella *et al.,* 1993). Clearly, additional biochemical analysis of the splicing activity is needed to understand the observed differences.

In the presence of the viral DNA genome, the preintegration complex forms a highly organized structure (Brown *et al.,* 1989) via interaction with the IN protomers (Ellison *et al.,* 1995). Hypothetically, the conformation or $K_m$ of this initial IN–viral DNA complex may be more stringent for initiating 3′-processing compared to strand-transfer activity. We speculate that while strand-transfer activity may not require the entire preintegration nucleoprotein complex, once formed, the complex may be maintained until the integration occurs. To what extent the LTR sequence contributes to complex formation in the 3′-processing and strand-transfer activities by IN is certainly of interest. Clearly, important questions regarding LTR substrate recognition by IN, LTR–IN complex formation, and processing mechanisms remain to be answered.

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