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The Genome of Moloney Murine Leukemia Virus Can Be Integrated by the Integrase of Human Immunodeficiency Virus Type 1 Expressed Alone *In vivo*

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ABSTRACT

An *in vivo* integration assay using the expressed human immunodeficiency virus type 1 (HIV-1) integrase (IN) protein and plasmids carrying a copy of the infectious Moloney murine leukemia virus (MuLV) provirus genome as substrates is presented. The HIV-1 IN gene was taken from vector pINSD and cloned into vector pXT1 to give pXT1-IN. Two and three nucleotides from the circle junction on one pair of U3 and U5 attachment (att) sequences on an infectious MuLV provirus vector pMLV-K were changed by means of site-directed mutagenesis to that of the corresponding HIV-1 att sequences to generate vector pMLV*(U3U5). The MuLV IN sequence was partially deleted for vectors pMLV-K and pMLV*(U3U5) to generate vectors pMLV Δ IN and pMLV*(U3U5) Δ IN. Integration of these wild type and MuLV IN partially deleted or att mutated MuLV provirus vectors in the transfected cells by the expressed HIV-1 IN was monitored by means of a non-radioactive reverse transcriptase (RT) assay for released and collected virions. No RT activity was detected for the NIH/3T3 cell singly transfected with vector pMLV Δ IN. However, some RT activities were observed for the HIV-1 IN expressing cell transfected either with vectors pMLV Δ IN or pMLV*(U3U5) Δ IN. This indicated that in the absence of other HIV-1 proteins expressed the MuLV provirus genome was integrated by the expressed HIV-1 IN protein. The integration of these MuLV provirus genomes was further confirmed by polymerase chain reaction analysis on the genomic DNA extracted from the transfected cells using the MuLV IN sequence remained from partial deletion as a target.

Key Words: retrovirus; integration; HIV-1 integrase.

I. Introduction

Integration of a DNA copy of the viral RNA genome into the genome of the host cell is one of an essential step in the retrovirus life cycle. The integration process consists of several steps: (1) 3' processing, which is the removal of two nucleotides from the 3' termini of the long terminal repeats (LTRs) (Bushman and Craigie, 1990; Collicelli and Goff, 1988; Krogstad and Champoux, 1990; LaFemina *et al.*, 1992; Roth *et al.*, 1989); (2) generation of a staggered cut on the host cell DNA, and (3) strand transfer, which is the joining of these 5' overhangs to the recessed viral DNA termini (Fujiwara and Mizuuchi, 1988; Katzman *et al.*, 1989; Vink *et al.*, 1991; Vora *et al.*, 1990). The joining reaction produces gaps which are repaired presumably by the host enzymes to create short duplications of the host sequences flanking the integrated provirus (Bushman and Craigie, 1991; Engelman *et al.*, 1991; Katz *et al.*, 1990; Sherman and Fyfe, 1990; Vink *et al.*, 1991). *In vitro* integration studies using bacterially expressed and purified human immunodeficiency

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virus type I integrase (HIV-1 IN) and synthetic oligonucleotides mimicking an inverted repeat region at both the 3' and 5' viral DNA ends, termed the attachment (att) sites, have demonstrated that IN is the only virally encoded protein that is able to perform all three activities described above (Craigie *et al.*, 1990; Katzman *et al.*, 1989). In addition, it has been shown that HIV-1 IN can also perform the reversal of the strand transfer reaction, termed disintegration, on a substrate that mimics a strand transfer intermediate (Chow and Brown, 1994; Chow *et al.*, 1992).

Both sequence alignment and mutational analysis of HIV-1 IN have identified three functional domains, namely, the HHCC "zinc finger" like sequence at the N-terminal region (Burke *et al.*, 1992; Bushman *et al.*, 1993; Johnson *et al.*, 1986; McEuen *et al.*, 1992), the so-called D, D(35)E central region (Bushman *et al.*, 1993; Drelich *et al.*, 1993; Engelman and Craigie, 1992; Kulkosky *et al.*, 1992; LeFemina *et al.*, 1992; van Gent *et al.*, 1992), and the nonspecific DNA binding region at the C-terminus (Puras *et al.*, 1994; Vink *et al.*, 1993; Woerner *et al.*, 1992; Woerner and Marcus-Sekura, 1993). Although the HHCC "zinc finger" may bind with zinc, *in vitro* binding assays have not detected any LTR-specific or nonspecific affinity for DNA in this region (Engelman and Craigie, 1992; Jonsson and Roth, 1993; Vincent *et al.*, 1993). Mutations at conserved histidine or cysteine residues in this motif impair both 3' processing and strand transfer but not the disintegration activities of HIV-1 (Engelman and Craigie, 1992; Leavitt *et al.*, 1993; Schauer and Billich, 1992; Vincent *et al.*, 1993), Rous sarcoma virus (RSV) (Bushman and Wang, 1994), or Moloney murine leukaemia virus (MuLV) IN's (Jonsson *et al.*, 1993). Possible involvement of this domain in earlier events in the viral life cycle has been addressed through *in vivo* mutational analyses on this region (Masuda *et al.*, 1995). However, the true function of the HHCC motif in the integration process has not been clearly defined (Masuda *et al.*, 1995). Three invariant amino acids, D64, D116, and E152, identified in the 4 central region, are also conserved across retrotransposons and prokaryotic transposases (Engelman and Craigie, 1992; Kulkosky *et al.*, 1992). Numerous mutational analysis using *in vitro* assays have defined this motif as the central catalytic domain of HIV-1 IN (Bushman *et al.*, 1993; Drelich *et al.*, 1992; Engelman and Craigie, 1992; Kulkosky *et al.*, 1992; LaFemina *et al.*, 1991; LaFemina *et al.*, 1992; Leavitt *et al.*, 1992; van Gent *et al.*, 1992). Noninfectious viruses have been produced by the mutations D116A and E152A and also by the V151D and E152Q double mutations (LaFemina *et al.*, 1992; Shin *et al.*, 1994). The carboxylic acid side chains of these acidic residues have been proposed to be involved in the coordination of divalent metal cations, such as Mg^{2+} or Mn^{2+}, required for the IN function (Kulkosky *et al.*, 1992). A putative leucine zipper motif has been predicted between residues V151 and Q168 of HIV-1 IN (Lin and Grandgenett, 1991; Wang *et al.*, 1994). Two-dimensional ^1H NMR and circular dichroism (CD) spectroscopy studies for a synthetic peptide corresponding to this region have indicated that the peptide adopts an amphipathic α-helical conformation in trifluoroethane containing solution (Cheng *et al.*, 1996). However, concentration-dependent CD studies have revealed that this peptide motif does not form dimers or oligomers in solutions as predicted (Cheng *et al.*, 1996; Wang *et al.*, 1994). These results are in agreement with the reported crystal structure of the catalytic domain of HIV-1 IN (Dyda *et al.*, 1994). The carboxy terminus of IN is the area of least sequence homology among different retroviruses (Cannon *et al.*, 1994; Vink *et al.*, 1993; Woerner *et al.*, 1992). This domain has been shown to be important for nonspecific binding of HIV-1 IN to DNA *in vitro* and; therefore, may interact with target DNA during DNA strand transfer (Cannon *et al.*, 1994). Recently, it has been found that virus assembly and maturation may be markedly affected by a H12N mutant near the N-terminus of HIV-1 IN (Engelman *et al.*, 1995). It also has been shown that truncated HIV-1 or MuLV IN's in different parts of the proteins can be mixed together to restore an intact IN function (Donzella *et al.*, 1996; Kalpana *et al.*, 1994; van Gent *et al.*, 1993).

Although different aspects of the influence of HIV-1 IN on the replication process of the virus have been identified through *in vivo* mutational analyses on the IN gene (Cannon *et al.*, 1994; Engelman *et al.*, 1995; Shin *et al.*, 1994), little is known about whether other viral or cellular components may play a role in the integration process *in vivo*. A human gene product that binds tightly to the HIV-1 IN *in vitro* and stimulates its DNA-joining activity has been investigated (Kalpana *et al.*, 1994). It is thought that the protein may encode a nuclear factor that promotes integration and targets viral DNA to active genes (Kalpana *et al.*, 1994). In this report, we present some experimental evidence to show that in the absence of any other HIV-1 proteins, the expressed HIV-1 IN can integrate the whole genome of MuLV *in vivo*. To use an infectious MuLV provirus vector pMLV-K as a substrate for expressed HIV-1 IN, we first partially deleted the MuLV IN sequence from the vector and then performed site-directed mutagenesis on one pair of the MuLV att sequences on the vector such that the last eight or nine bp from the circle junction (CJ) of the U3 or U5 LTR's were exactly similar to those of the corre-

sponding HIV-1 sequences. Mutated or wild type infectious pMLV-K vectors were then used to transfect a HIV-1 IN expressed and drug selected mouse fibroblast cell line NIH/3T3. Virions released from such transfected cells were collected and assayed using a non-radioactive reverse transcriptase (RT) activity assay method for the MuLV RT. We also performed PCR analysis on the genomic DNA extracted from each transfected cell to examine whether the viral genome was integrated. Based on these results, we found that, in the absence of MuLV IN, both the wild type or att sites mutated MuLV provirus genomes could be integrated equally well by the expressed HIV-1 IN. Comparison of these findings with those of the *in vitro* integration assay results is included herein.

II. Materials and Methods

1. Materials, Bacterial Strains, and Cell

[^{35}S]dATPαS (600 Ci/mmol) was purchased from Amersham. Unless otherwise stated, all the restriction enzymes were purchased from New England Biolabs. T4 DNA ligase and alkaline phosphatase were obtained, respectively, from GIBCO BRL and Boehringer Mannheim. T4 DNA polymerase was purchased from Boehringer Mannheim. Bacteria strains HB101 and AG-1 for DNA amplification were purchased from the Culture Collection and Research Center, Taiwan, (CRCC) and Stratagene, respectively. NIH/3T3 (embryo, contact-inhibited, NIH Swiss mouse) cells were obtained from the American Type Culture Collection (ATCC). Cells were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium (D-MEM), supplemented with 10% calf serum and sodium bicarbonate to pH 7.2 (Cone and Mulligan, 1984).

2. Vector Construction

The HIV-1 IN expression vector pXT1-IN was constructed from the plasmids pXT1 (Boulter and Wagner, 1987) and pINSD (Bushman and Craigie, 1991; Bushman *et al.*, 1993; Engelman and Craigie, 1992) (Fig. 1A). Key genetic elements on the eukaryotic expression vector pXT1 obtained from Stratagene were 2 LTR's of MuLV, a neomycin resistant gene, and a herpes simplex thymidine kinase (TK) promoter for controlling the expression of the cloned gene (Boulter and Wagner, 1987). Plasmid pINSD with a copy of the intact HIV-1 IN gene inserted on it was obtained from Duane Grandgenett of the St. Louis University Medical Center. Both plasmids were linearized with *Bgl* II digestion and then ligated together with T4 DNA ligase to generate plasmid pXT1-IN-p1, which was approximately 16,000 bp in size. The excessive pINSD sequence on this plasmid was further removed through a series of enzymatic digestions, filling-up and ligation reactions to give the HIV-1 IN expression vector pXT1-IN (Fig. 1A).

Plasmid pXT1 was shortened with *Xba* I digestion and then T4 DNA ligation to generate a plasmid

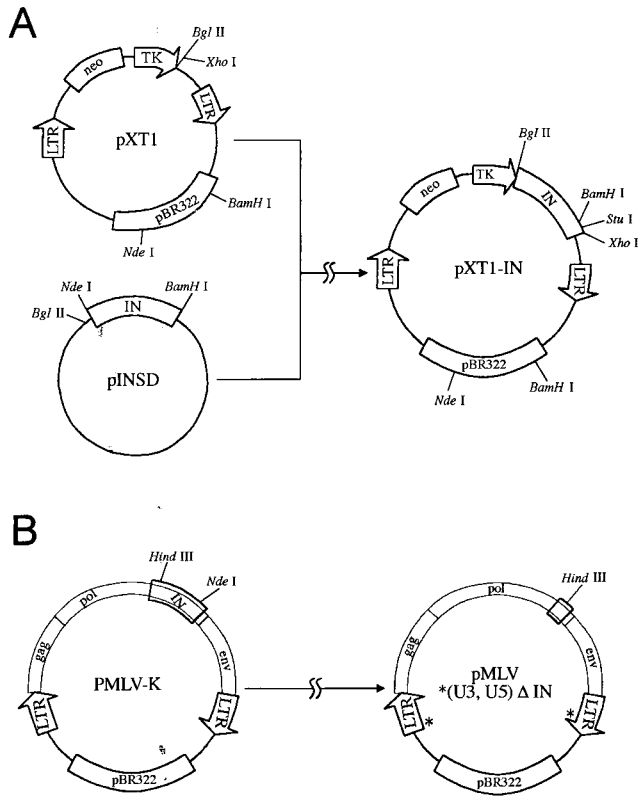


Fig. 1. (A) Construction of vector pXT1-IN from plasmids pXT1 and pINSD. Plasmids pXT1-IN and pINSD were linearized by *Bgl* II digestion first and then ligated together using the T4 DNA ligase to generate plasmid pXT1-IN-p1. Using partial *Bam* HI and complete *Xho* I digestions, Klenow filling-up, and ligation of the blunted DNA with T4 DNA ligase, the excessive original pINSD sequence on the plasmid was removed to create plasmid pXT1-IN-p2. To generate the HIV-1 IN expression vector pXT1-IN, the other excessive pINSD sequence on plasmid pXT1-IN-p2 was further removed through *Bgl* II and *Nde* I digestions, Klenow filling-up, and blunt-end ligation by T4 DNA ligase. (B) Construction of vector pMLV*(U3U5) Δ IN from plasmid pMLV-K. Plasmid pMLV-K was digested with *Nhe* I to give two fragments, and the larger one (8, 264 bp) was ligated with the *Nhe* I digested and alkaline phosphatase treated plasmid pLTR*(1U3,1U5) to generate plasmid pMLV*(U3U5). The construction of plasmid pLTR*(1U3,1U5) is described in the text. The MuLV IN sequence on plasmid pMLV*(U3U5) was deleted through *Hind* III and *Nde* I digestions, Klenow filling-up, and T4 DNA ligation to generate plasmid pMLV*(U3U5) Δ IN.

with only one LTR on it. Site-directed mutagenesis was then performed on the U3 and U5 att sequences of the one LTR containing plasmid to give plasmid pBR322-LTR*(1U3,1U5) (6,419 bp). This plasmid was truncated further with *Hind* III and *Bam*H I digestions, Klenow filling-up, and then ligation with T4 DNA ligase to create plasmid pLTR*(1U3,1U5) (6,073 bp). The infectious MuLV provirus vector pMLV-K (14,150 bp) was obtained from Dusty Miller of the Fred Hutchinson Cancer Research Center (Miller and Verma, 1984) (Fig. 1B). The vector was digested with *Nhe* I to give two fragments, and the larger one (8,264 bp) was ligated with the *Nhe* I digested and alkaline phosphatase treated pLTR*(1U3,1U5) to generate plasmid pMLV*(U3U5) (14,337 bp). Deletion of the MuLV IN sequence on the plasmid was performed to give plasmid pMLV*(U3U5) Δ IN (13,830 bp) (Fig. 1B). Plasmid pMLV Δ IN was made by replacing the portion of the mutated att sites on each LTR plus the entire pBR322 sequence of plasmid pMLV*(U3U5) Δ IN with that of the same sequence of plasmid pMLV-K through *Nhe* I digestion and then ligation with the T4 DNA ligase.

3. Site-Directed Mutagenesis

A transformer site-directed mutagenesis kit purchased from Clontech was used to mutate a pair of wild type MuLV att sequences to those of the corresponding HIV-1 sequences, namely, from AATGAAAG to ACTGGAAG and from TCTTT CAT to TCTAGCAG, on one U3 and on one U5 of each LTR, respectively. The sequences for mutagenic primers pXT1-IN-U3 and pXT1-IN-U5 were 5'-GAAAAAGGGGGG-ACTGGAAGACCCC ACCTC and 5'-CAGCGGGGGTCTAGCAGTT GGGGGCTCGTC, respectively. The method (Perlak, 1990) also required a selection primer pXT1-IN-sel, and the corresponding sequence was 5'-GAGTGCACCAAATGCGGTGTG. All the oligonucleotides were synthesized by DNAFax Taiwan. The experimental procedures described in the manufacturer's manual were followed.

4. Transfection and Infection of Cell

Cells were plated at 5×10^5 cells per cm^2 one day prior to transfection. Using the Promega (Madison, Wisconsin) transfectam reagent for the transfection of eukaryotic cells, 10 μg of plasmid pXT1-IN or pMLV-K or each of its derivatives (all in closed circular form) was transfected into cells. After 4 h of reaction, the cells were washed twice with 5ml of 1 × phosphate-buffered saline (PBS). The cells were then grown in 10 ml fresh medium at 37 °C and in 5% CO_2 for 48 h before 400 μg/ml G418 was added for selection (for the pXT1-IN transfected cells only). To examine the presence of virions, culture supernatants were collected at various time periods 48 h posttransfection. The supernatants were filtered through 0.45 μm-pore-size nitrocellulose, and equal RT activity of wild type and mutant MuLV virions was used for infection. Each infection mixture contained 3 ml of filtered virions and 4-8 μg/ml polybrene (Lynch and Miller, 1991; Miller and Verma, 1984). The infection steps were performed on $1.0\text{-}1.2 \times 10^6$ cells in a 100 mm dish for 90-120 min. Then, one-tenth or one-twentieth of the original amounts of cells was subcultured 24 h postinfection for drug selection.

5. RT Activity Assay

A non-radioactive RT activity assay kit purchased from Boehringer Mannheim was used for RT activity assay for MuLV RT. Virions were collected from culture supernatants through ultracentrifugation at 40,000 × g for 1.5 h at 4 °C. Collected virions were lysed with 40 μl of lysis buffer (50 mM Tris buffer at pH 7.8 containing 80 mM KCl, 2.5 mM dithiothreitol, 0.75 mM EDTA, and 0.5% Triton X-100). Then, the RT assay procedures described by the manufacturer were followed. The RT assay procedures were repeated three times for virions collected from three different transfection experiments. However, only one RT assay was performed for virions collected from each infection experiment. The averaged RT signal measured for the background was 0.098.

6. DNA Sequencing and PCR Analysis

The deleted MuLV IN and mutated MuLV att sequences at one U3 and one U5 on each LTR were sequenced using primers Seq-MuLV-IN; 5'-CAG TCCC TACTACATGCTG, Seq-U3; 5'-GCCTATA GAGTA CGAGCC, and Seq-U5; 5'-CGCTGTT CCTTGGG AGGG, respectively. Sequencing was performed with a Sequenase V2.0 kit as instructed by the manufacturer (US Biochemical). To extract genomic DNA from cells grown in confluence, procedures described in standard molecular cloning protocols were followed. Three groups of primers were designed for PCR analysis on the extracted genomic DNA, namely, (1) primers designed for detection of deleted and remaining MuLV IN sequence from partial deletion: MLV-IN-3'; 5'-GGAATTCCGGCGTGTTGCGGGC TCGG, MLV-IN-5'; 5'-GGAATTCCGGGAA CTAGGG-TCCG CGGG, MLV-IN-sense; 5'-GAGAGAAGCCA CAGTCCC, and MLV-IN-antisense; 5'-GGTAGGC

TGCCGCCAGAGG; (2) primers designed for detection of HIV-1 IN sequence: HIV-1-IN-3'; 5'-GGAATT CCGGGGATT-GTAGGG AATGCC and HIV-1-IN-5'; 5'-GGAATTCCGGCCC AAGAAGAACA TGAG; and (3) primers designed for detection of various parts of MuLV genome: MLV-PR-3'; 5'-CAACAC TTGCAGGGGCTGCCCC, MLV-PR-5'; 5'-GGG AGGTCAGGGTCAGGAGC, MLV-RT-3'; 5'-GGAA TTCCGGTGGGGTGGAG TCT-CAGGC, MLV-RT-5'; 5'-GGAATTCCGGCC TGGGCGGAAACCGG, MLV-p10-3'; 5'-GGTCCC CGAGGTCCTCGTGG, MLV-p30-5'; 5'-GCGGGTC TCCAAAACGCGGGC, MLV-gp70-3'; 5'-GGGCTT CCAGTAAGCTCTA CCGG, and MLV-gp70-5'; 5'-GCTTCGCCCGGC TCCAGTCC. Each PCR reaction was performed using 30 cycles of amplification, each consisting of a denaturation step at 94 °C for 4 min followed by an annealing-extension step at 65 °C for 1 min and at 70 °C for 30 sec to 1 min (Silver and Keerikatte, 1989). The annealing-extension step used for primers MLV-IN-sense and MLV-IN-antisense was fixed at 70 °C for 15 sec only. The total volume for each PCR reaction was 50 μl. Each reaction contained 2.5 units of DNA polymerase (DynaZyme II, Finnzymes Oy), PCR buffer (10mM Tris-HCl, pH8.8, 1.5 mM $MgCl_2$, 50mM KCl, and 0.1% Triton X-100), dNTP mixture (dATP, dGTP, dCTP, and dTTP, each 250 μM), 0.2 μM sense primer, 0.2 μM antisense primer, and 100 ng plasmid or 2.5 μg genomic DNA. Amplified products were resolved on an agarose gel and visualized by means of ethidium bromide (EtBr) staining.

7. Western Blot Analysis

The anti-HIV-1-IN antisera (#104103 rabbit antisera) was obtained from Duane Grandgenett of the St. Louis University Medical Center. Protein samples collected from lysed cells were subjected to SDS-12.5% polyacrylamide gel electrophoresis (SDS-PAGE). After blotting the proteins onto a nitrocellulose membrane (Hybond-ECL, Amersham), we incubated the membrane with the anti-HIV-1-IN antisera and then incubated it with horse radish peroxidase-conjugated goat-anti-rabbit immunoglobulin, respectively. The presence of HIV-1 IN protein was visualized by means of a color staining reaction using 4-chloro-1-naphthol.

III. Results

1. Establishment of a Stable HIV-1 IN Expression Cell Line and Partial Deletion of MuLV IN Sequence from MuLV Provirus Genome

Our strategy for constructing the *in vivo* integration assay was to establish a stable HIV-1 IN expression cell line first. The expression of HIV-1 IN was controlled by a TK promoter on the constructed pXT1-IN vector (Boulter and Wagner, 1987). A stable HIV-1 IN expressing cell line was obtained after about 30 days of G418 selection of the pXT1-IN transfected NIH/3T3 cells. Western blot analysis of the total cell lysate (Fig. 2, lanes 3-5) revealed an immunoreactive protein at the expected molecular mass of a bacteria expressed and purified protein. However, it appeared that some proteins were degraded either inside the cells or during the processing for Western blot analysis (Fig. 2, lanes 3-5). Once a stable HIV-1 IN expressing cell line was established, a series of wild type or MuLV IN partially deleted and att sequences mutated infectious MuLV provirus vectors was used to transfect the cells and drug selection by G418 was continued. The MuLV IN is composed of 404 amino acid residues (Jonsson *et al.*, 1986; Jonsson

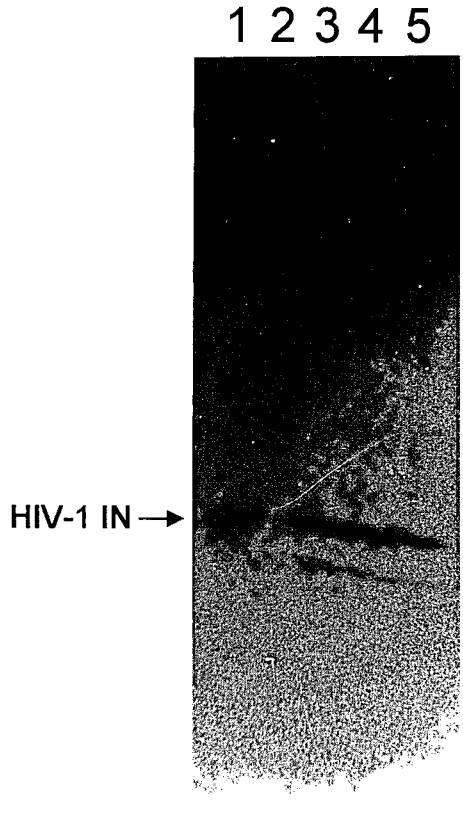


Fig. 2. Western blot analysis of HIV-1 IN expressed in NIH/3T3 cells. The expression of HIV-1 IN protein (lanes 3, 4, and 5) was visualized by means of a color staining reaction using 4-chloro-1-naphthol. Lane 1 was a positive control for the HIV-1 IN, purified from vector pINSD transformed bacteria. Lane 2 was a negative control prepared from the untransfected NIH/3T3 cells.

et al., 1996; Kulkosky *et al.*, 1992). The central catalytical residues or the so-called D, D(35)E motif identified for the MuLV IN were Asp^{125}, Asp^{184}, and Glu^{220}, respectively (Jonsson *et al.*, 1996; Kulkosky *et al.*, 1992). Through digestion with a *Hind* III site at 4894 bp and a *Nde* I site at 5401 bp on the MuLV genome, the total number of residues removed was 169. This included the entire central catalytic domain plus some flanking sequences of the MuLV IN. Therefore, the partially deleted MuLV IN completely lost its catalytic activities for 3' processing and DNA strand transfer. This deletion was confirmed by means of DNA sequencing (Fig. 3A). This ensured that integration of the MuLV genome was performed only by the expressed HIV-1 IN protein in this system.

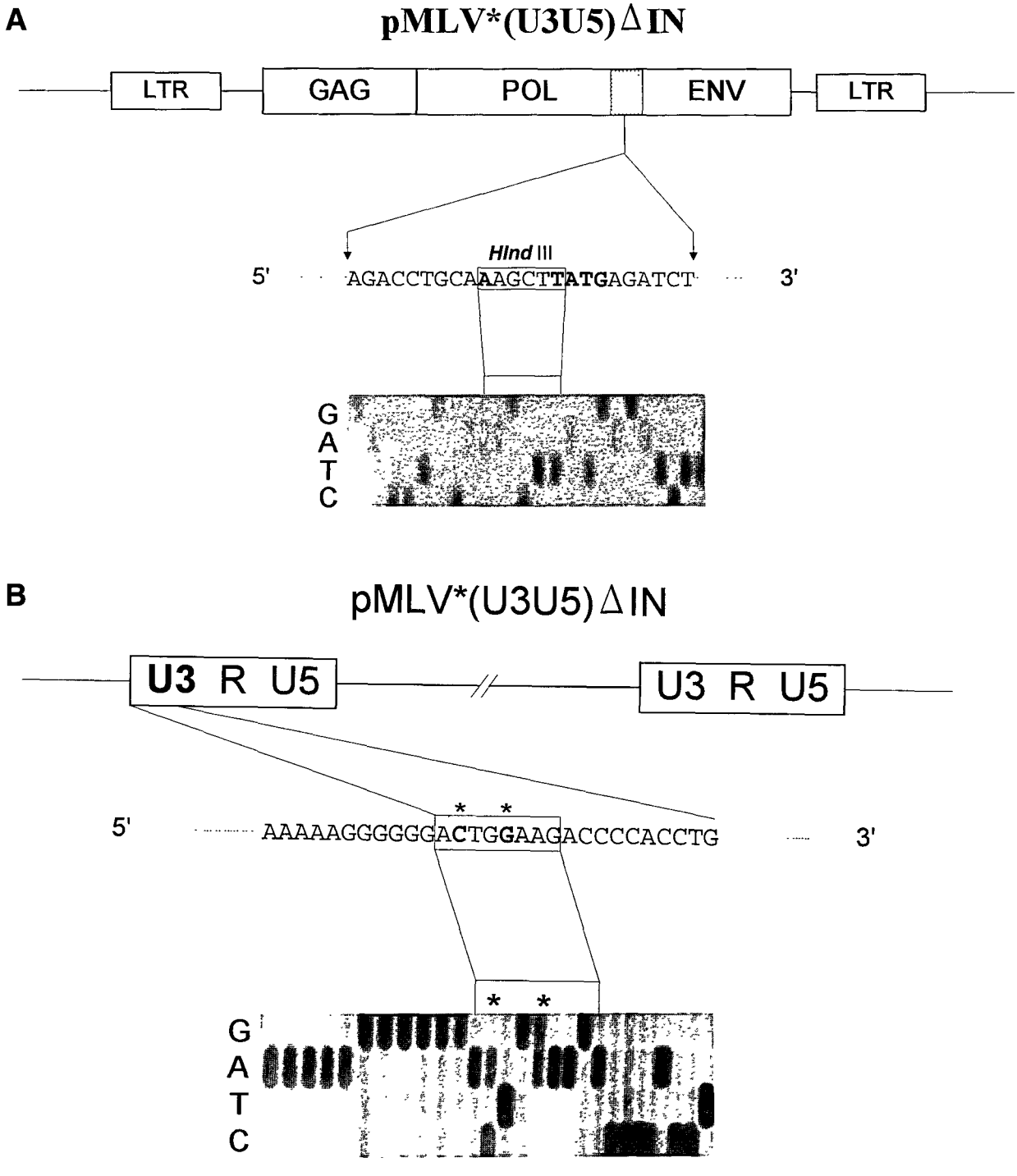
2. Mutation of MuLV Att Sequences on a Pair of U3 and U5 LTR's on MuLV Provirus Vectors

The infectious pMLV-K plasmid which contained a copy of the full MuLV provirus genome was made by Miller and Verma from noninfectious pMLV-1 and infectious pMLV-48 clones (Miller and Verma, 1984). Infectious progeny virus particles were produced and detected by XC plaque assay through transfection of the NIH/3T3 cell with the pMLV-K vector (Lynch and Miller, 1991; Miller and Verma, 1984). Since each LTR sequence on the pMLV-K vector was composed of U3, R, and U5 sequences in tandem, the total number of pairs of att sequences on the vector was two. Reverse transcription of viral RNA to DNA required a cellular 4S transfer RNA (tRNA) as a primer. The tRNA is intimately associated with a primer binding site (PBS) located immediately adjacent to the U5 att sequence (Levy, 1993). Reverse transcription is initiated at the PBS and directed across the att sequence toward the 5' end of the viral genome (Levy, 1993). The reverse transcription process is stalled if the PBS sequence is altered (Murphy and Goff, 1989). To avoid possible interruption in the reverse transcription, we chose to mutate only one pair of att sequences. The first mutated att sequence was in the U3 region at the 5' end of the genome while the second mutated one was in the U5 region at the 3' end of the genome (Fig. 3B and 3C). These mutated att sequences may not be incorporated into a progeny particle if transcription of the provirus genome by cellular RNA polymerase to a 5' capped mRNA is initiated at the 5' R rather than at the 5' mutated U3 region. The extent of such a mutation of each att sequence can not be too long since there are complex secondary RNA structures around the PBS and att sequences which are essential for efficient initiation of reverse transcription (Aiyar *et al.*, 1994; Cobrinik *et al.*, 1988). However, Bushman and Craigie (1990) and Vink *et al.* (1991) have reported that the terminal nine bp of U3 and the terminal fifteen bp of U5 att sequences are important for both specific cleavage and integration by HIV-1 IN *in vitro*. The most critical region for HIV-1 IN to exert has been identified as being between nucleotides two and nine (Bushman and Craigie, 1991) while that identified for MuLV IN is between nucleotides three and seven from the CJ (Bushman and Craigie, 1990). If we mutated a A and also a A at the second and fifth positions to a C and a G from the CJ, respectively, for the MuLV U3 att sequence, the sequence of the last eight nucleotides from the CJ was identical to that of the corresponding sequence of HIV-1 (Fig. B). Likewise, if the second, fifth, and sixth nucleotides of the MuLV U5 att sequence were mutated from T to G, T to G, and T to A, respectively, the total number of nucleotides from the CJ that was identical to the corresponding HIV-1 sequence was nine (Fig. 3C). It was necessary to make a compromise so that the extent of mutation was not be so long as to interfere with the reverse transcription or so short as to affect the integration by expressed HIV-1 IN. However, other *in vitro* studies have shown that the location of the 3' cleavage is dependent on the position of the conserved CA dinucleotide and is not defined by a fixed distance from the CJ (Bushman and Craigie, 1991; Leavitt *et al.*, 1992; Vink *et al.*, 1991). Since only one pair of att sequences were mutated, the sequences of the unmutated pair also appeared on the sequencing gel (Fig. 3B and 3C).

3. RT Activity Assay for Virions Released

To investigate whether HIV-1 IN expressed alone could integrate the MuLV genome *in vivo*, we transfected a series of MuLV IN partially deleted and att sequence mutated or unmutated infectious pMLV vectors, namely, pMLV*(U3U5) Δ IN, pMLV*(U3,U5), and pMLV Δ IN to the HIV-1 IN expressing NIH/3T3 cell. Since no endogenous MuLV IN gene was detected through PCR analysis on the genomic DNA extracted from the original untransfected cell (Fig. 6), the RT activity assayed for produced progeny particles could be attributed to successful integration of MuLV genome by the HIV-1 IN expressed in the cell. For cells singly transfected with vectors pMLV-K, pMLV*(U3U5), pMLV Δ IN, or pMLV*(U3U5) Δ IN, drug selection was not necessary since no selection marker was engineered on these vectors. No apparent RT activity was detected for cells singly transfected with vectors pMLV Δ IN or pMLV*(U3U5) Δ IN (Fig. 4A and 4B). However, the RT activity increased substantially when each of these two vectors was used to transfect the HIV-1 IN expressing cell (Fig. 4A and 4B).

Except for the case of transfection of the wild type (WT) pMLV vector, pMLV-K, to the HIV-1 IN expressing cell, the RT activities detected for cells singly transfected with vectors pMLV-K or pMLV*(U3U5) were both considerably larger than those detected for vectors pMLV Δ IN and pMLV*(U3U5) Δ IN transfected and the HIV-1 IN expressed cells. This indicated that while the production of the virus might be somewhat affected by the efficiency of transfection of a vector into a drug selected cell, the major cause of reduction in virus production was the change in integration efficiency. As expected, the efficiency of integration by HIV-1 IN on the MuLV genome was much lower than that by MuLV IN on its own sequence (Fig. 4A). It appeared that whether or not the att sequences were mutated was not critical to the integration efficiency of



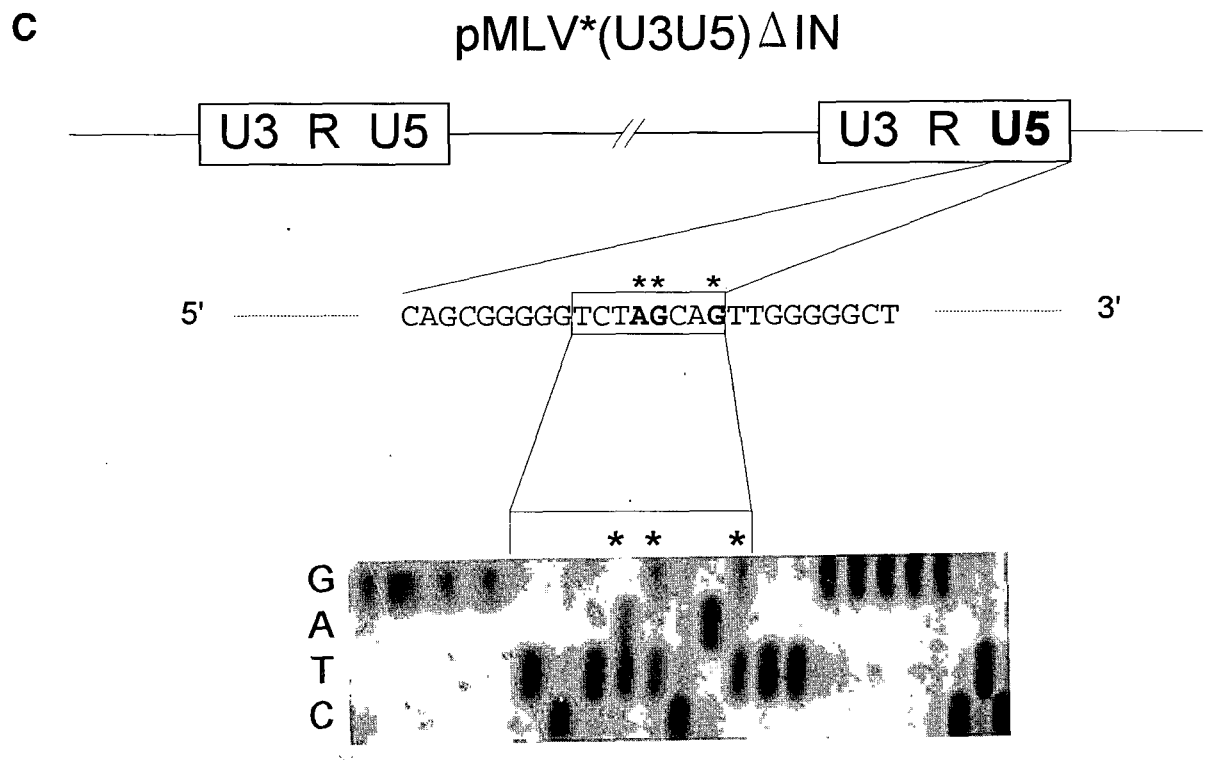


Fig. 3. DNA sequencing for the partially deleted MuLV IN and mutated MuLV att sequences at one U3 and one U5 LTR of vector pMLV*(U3U5)ΔIN. (A) The sequence of partially deleted MuLV IN was presented as the sequence at the junction of the restored *Hind* III and the partially destroyed *Nde* I sites after deletion of the partial MuLV IN sequence and ligation of the remaining sequence. The original and mutated nucleotides and their positions from the MuLV circle junction (CJ) were as follows:

original			mutated		
U3	CJ	U5	U3	CJ	U5
TCTTTCATT	|	AATGAAAGAC	TCTTCCAGT	|	ACTGCTAGAC
AGAAAGTAA	|	TTACTTTCTG	AGAAGGTCA	|	TGACGATCTG
			8 7 6 5 4 3 2 1		1 2 3 4 5 6 7 8 9

After these mutations, 8 nucleotides from the CJ of the U3 or 9 nucleotides from the CJ of the U5 att sequences were exactly similar to those of the corresponding HIV- sequences. Since there were two pairs of U3 and U5 att sequences on the provirus genome and only one pair was mutated, the sequences of the unmutated pair also appeared on the sequencing gel. (B) The sequence of the mutated U3 att site and (C) The sequence of the mutated U5 att site.

HIV-1 IN on the MuLV genome since the RT activities detected for the vectors pMLV Δ IN or pMLV*(U3U5) Δ IN transfected and the HIV-1 IN expressed cells were nearly the same with each other (Fig. 4A and 4B). The residual integration activities of these two cases accounted for about 15% of the peak value of the case of transfection of the NIH/3T3 cell using the pMLV-K vector (Fig. 4A). Similar to other *in vivo* integration studies using mutated HIV-1 att sequences (Masuda *et al.*, 1995), our data also showed that the mutated att sequences affected integration less severely than might have been expected from the results of various *in vitro* integration studies, in which att site mutants showed considerably less integration (Chow *et al.*, 1994; Chow *et al.*, 1992; LaFemina *et al.*, 1991; Leavitt *et al.*, 1992; Vink *et al.*, 1991). In fact, HIV-1 IN was found to not be able to cleave the MuLV U5 oligonucleotide with high specificity (Vink *et al.*, 1991). To examine whether infectious progeny particles could be produced by cells containing the integrated MuLV genome by the expressed HIV-1 IN, we infected new NIH/3T3 cells with virions collected from culture supernatants for the HIV-1 IN expressing cells transfected with vectors pMLV Δ IN or pMLV*(U3U5) Δ IN. RT activities measured for these two cases plus that measured for cells infected with virions generated by vector pMLV-K transfected cells are presented in Fig. 5. Note that the RT activity measured for virions produced in the pMLV*(U3U5) Δ IN case was larger than that measured for virions produced in the pMLV Δ IN case.

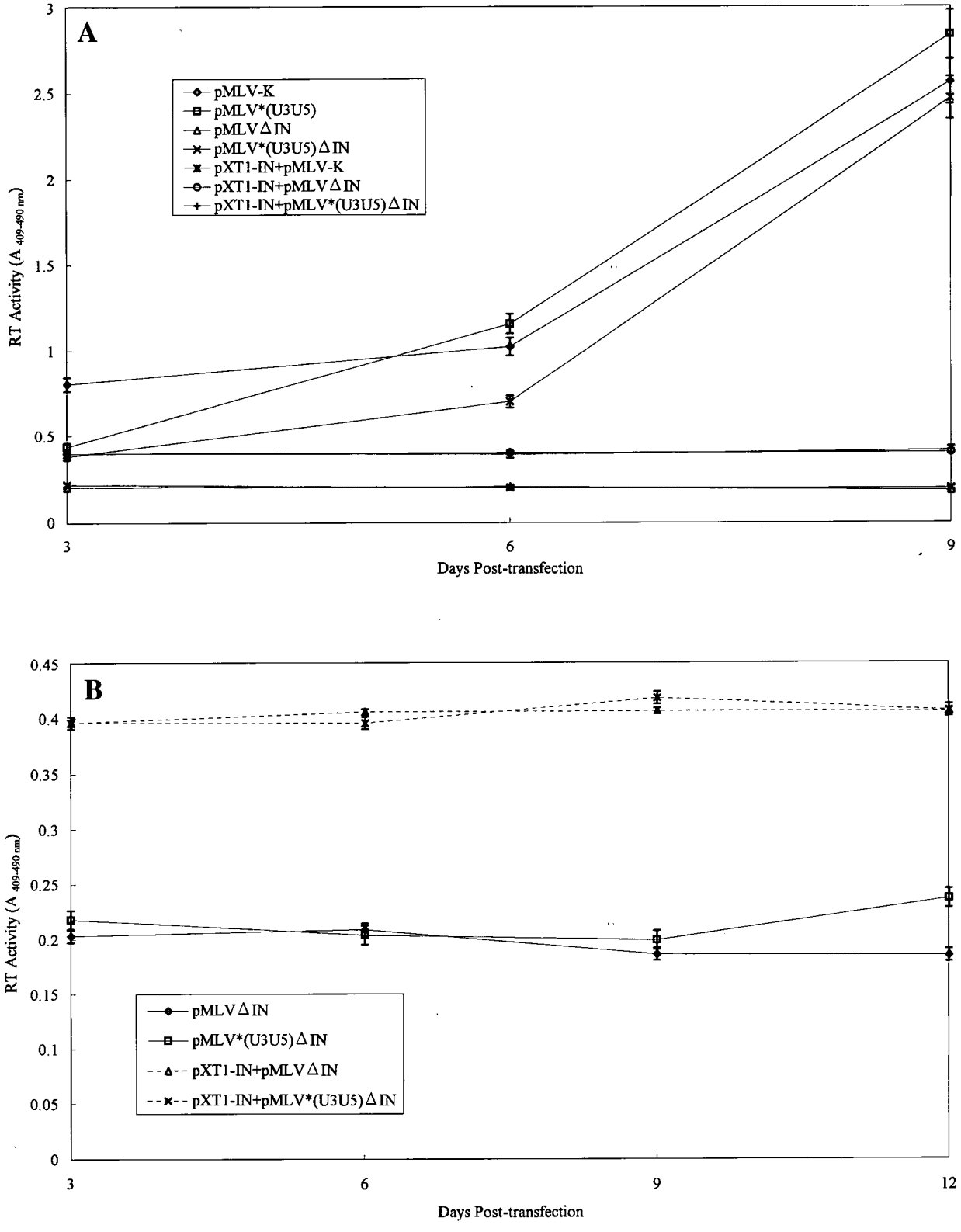


Fig. 4. RT activity assay for virions released from the transfected cells. (A) RT activities measured for the NIH/3T3 cells transfected with a series of wild type, att sites mutated, or MuLV IN partially deleted MuLV provirus vectors. Symbols used to designate the signals of the RT activity measured for each case are depicted in the inset. (B) Amplified plots for RT activities measured for the NIH/3T3 cells transfected with vectors pMLV Δ IN and pMLV*(U3U5) Δ IN, or for the HIV-1 Δ IN expressing cells transfected with vectors pMLV ΔIN and pMLV*(U3U5) Δ IN. Symbols used to designate the signals of the RT activity measured for each case are also depicted in the inset.

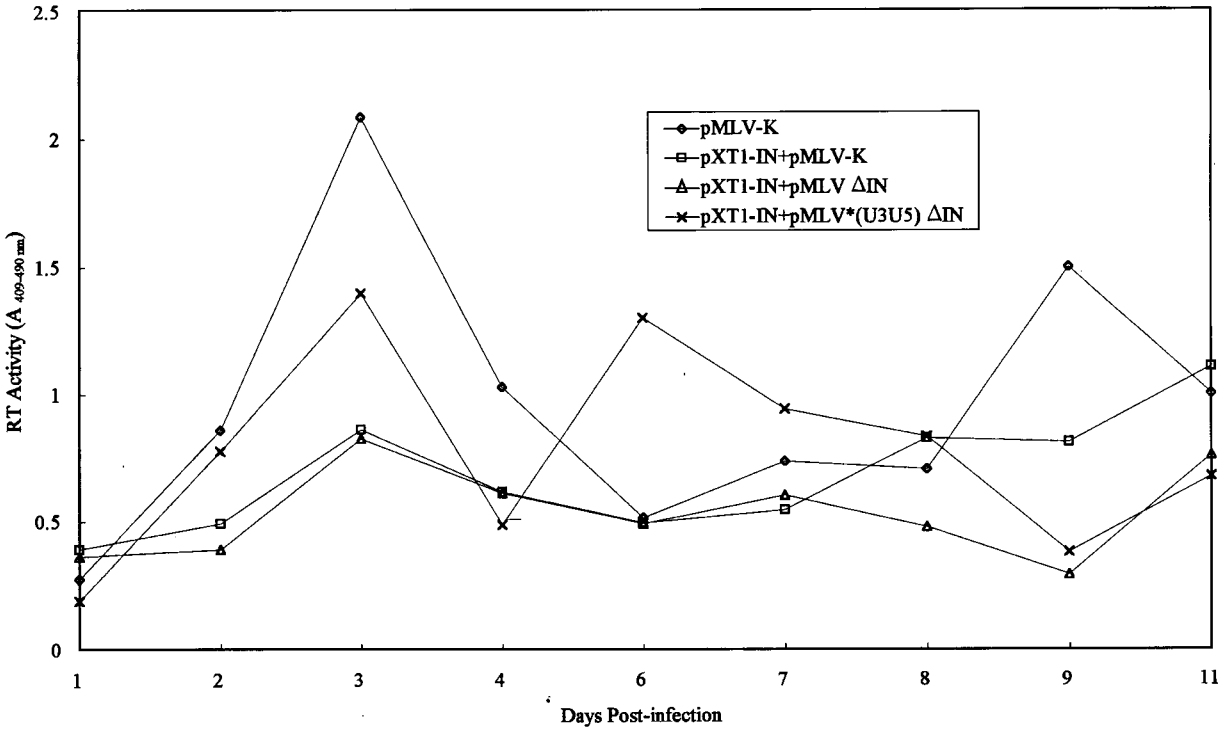


Fig. 5. RT activity assay for virions released from the infected cells. Virions collected from culture supernatants of the transfected cells were filtered, and equal RT activity of wild type and mutant MuLV virions was used to infect the NIH/3T3 cells. After 24 h of each infection process, one-tenth or one-twentieth of each original amount of cells was subcultured for drug selection or for RT activity measurement. The same RT activity assay kit used in the transfection case was used for the RT activity assay. Again, the symbols used to designate the signals of RT activity measured in each case are depicted in the inset.

Since whether or not the MuLV att sequences were mutated was not critical to the activity of expressed HIV-1 IN (Fig. 4A and 4B) and since equal RT activity of virions was used in each infection, the difference in the RT activities measured in these two cases might be explained by the fact that different amounts of expressed HIV-1 IN proteins were incorporated into the progeny particles. It could be also due to the fact that cells infected with virions produced by the expressed HIV-1 IN and vector pMLV Δ IN or pMLV*(U3U5) Δ IN transfected cells could not be selected by G418 while that infected with virions produced from the expressed HIV-1 IN and vector pMLV-K transfected cell could be selected by G418.

4. PCR analysis on the Genomic DNA Extracted

To further confirm the integration of MuLV genome by the expressed HIV-1 IN, we designed a PCR analysis strategy for genomic DNA extracted from HIV-1 IN expressing cells transfected with vector pMLV-K, pMLV Δ IN, or pMLV*(U3U5) Δ IN. First, primers that could be bound on the MuLV IN, RT, PR, P10 or P70 regions were used to examine whether there were corresponding endogenous sequences present in the original NIH/3T3 cell. We found that endogenous sequences were detected for all of the above regions except the IN one (Fig. 6). Therefore, the IN region was chosen as a target for examining whether the MuLV genome was integrated. We designed two pairs of primers with were both aimed at the IN region. The binding positions of the first pair were placed inside the sequence that was removed by *Hind* III and *Nde* I digestions. Using these primers for PCR analysis on the IN undeleted and integrated MuLV genome, a 453 bp DNA band could be generated and seen on a resolving agarose gel. This was observed for the cells singly transfected with vector pMLV-K or for the HIV-1 IN expressing cells transfected with the same vector (Fig. 7). Next, a pair of primers that could detect the remaining MuLV IN sequence from partial deletion of MuLV IN of the integrated MuLV provirus genome was designed. The 5' and 3' binding positions of these primers were placed, respectively, in a region between the N-terminal of IN and the *Hind* III site and in a region between the *Nde* I site and the C-terminal of IN (Fig. 8). Using these primers for PCR analysis on the IN partially deleted and integrated MuLV provirus genome, a 222 bp DNA band could be generated

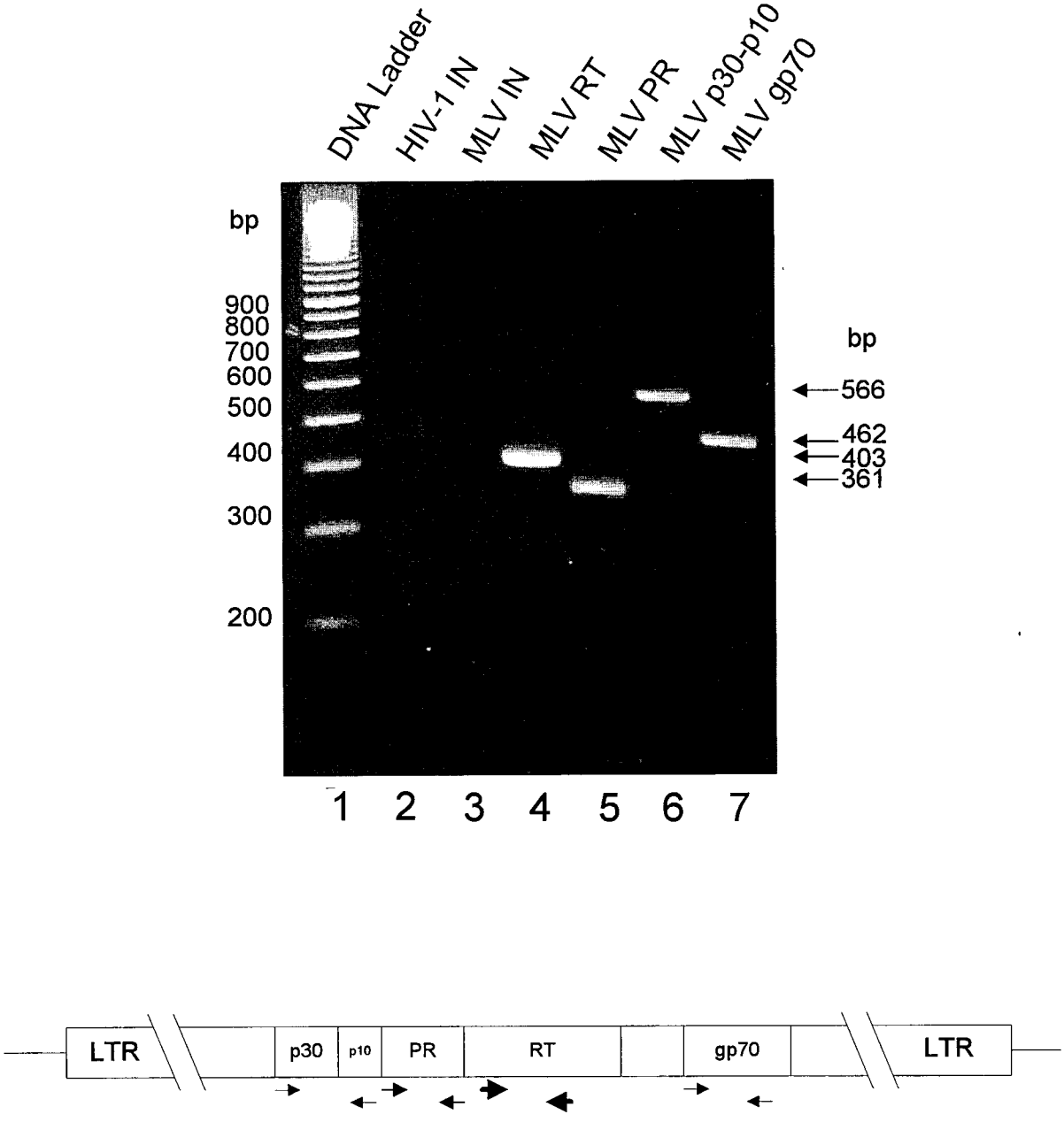


Fig. 6. PCR analysis of the genomic DNA extracted from NIH/3T3 cells. Six primer sets were used in this analysis, and the sequences of these primers are described in the text. Lane 1 was a DNA ladder; lane 2, PCR analysis on the genomic DNA extracted from the NIH/3T3 cells using primers designed for detection of the HIV-1 IN sequences; lane 3, PCR analysis of the same genomic DNA extracted from the NIH/3T3 cells using primers designed for detection of the MuLV IN sequence; lanes 4-7, PCR analysis of the genomic DNA extracted from the NIH/3T3 cell using primers designed for detection of various parts of MuLV genome. The binding positions of each of these primer sets are depicted the diagram below the agarose gel used to resolve the generated PCR products.

and seen on a resolving agarose gel. This was indeed observed for HIV-1 IN expressing cells transfected with either vectors pMLV Δ IN or pMLV*(U3U5) Δ IN (Fig. 8).

IV. Discussion

We have shown that in the absence of other HIV-1 proteins, the HIV-1 IN expressed alone in the NIH/3T3 cell could integrate the whole infectious MuLV provirus genome *in vivo*. The efficiency of this integration was about 15% of that performed by the MuLV IN on the WT MuLV provirus genome. This efficiency was found to not be affected by whether or not one pair of att sequences (two or three nucleotides of U3 or U5 att sequences from the CJ) was partially mutated.

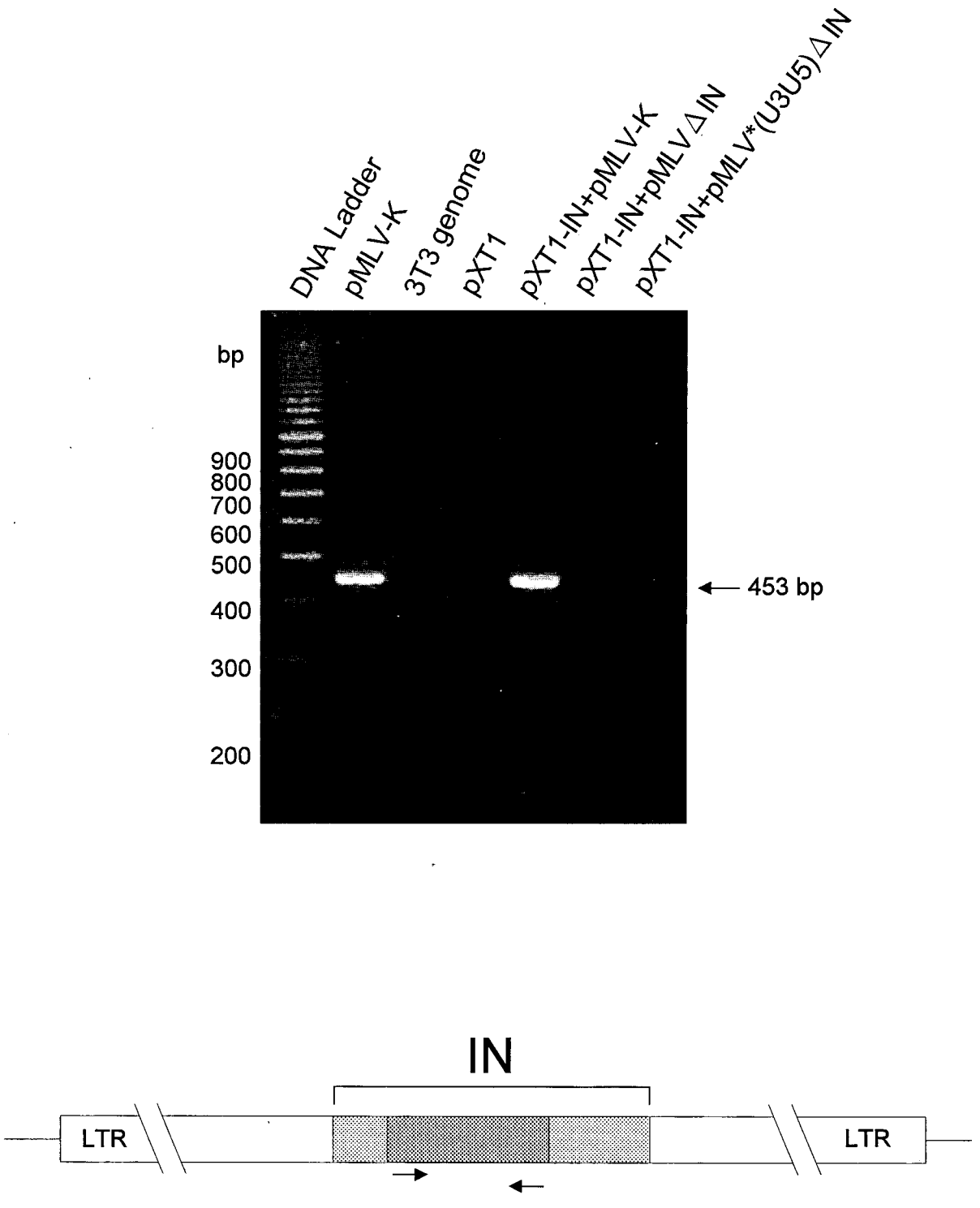


Fig. 7. PCR analysis of the genomic DNA extracted from the NIH/3T3 cells transfected with a variety of vectors or HIV-1 IN expressing cells transfected with vectors pMLV-K, pMLV Δ IN, or pMLV*(U3U5) Δ IN using a primer set that could detect the portion of the partially deleted MuLV IN sequence. The binding positions of these primers are depicted in the diagram below the agarose gel used to resolve the PCR generated products. Lane 1 was a DNA ladder; lane 2, PCR analysis on the genomic DNA extracted from vector pMLV-K transfected NIH/3T3 cells; lane 3, PCR analysis on the genomic DNA extracted from NIH/3T3 cells; lane 4, PCR analysis on the genomic DNA extracted from vector pXT1 transfected NIH/3T3 cells; lane 5, PCR analysis on the genomic DNA extracted from the HIV-1 IN expressing cell transfected with vector pMLV-K; lanes 6 and 7 were PCR analysis results for the genomic DNA extracted from the HIV-1 IN expressing cells transfected with vector pMLV Δ IN or pMLV*(U3U5) Δ IN, respectively.

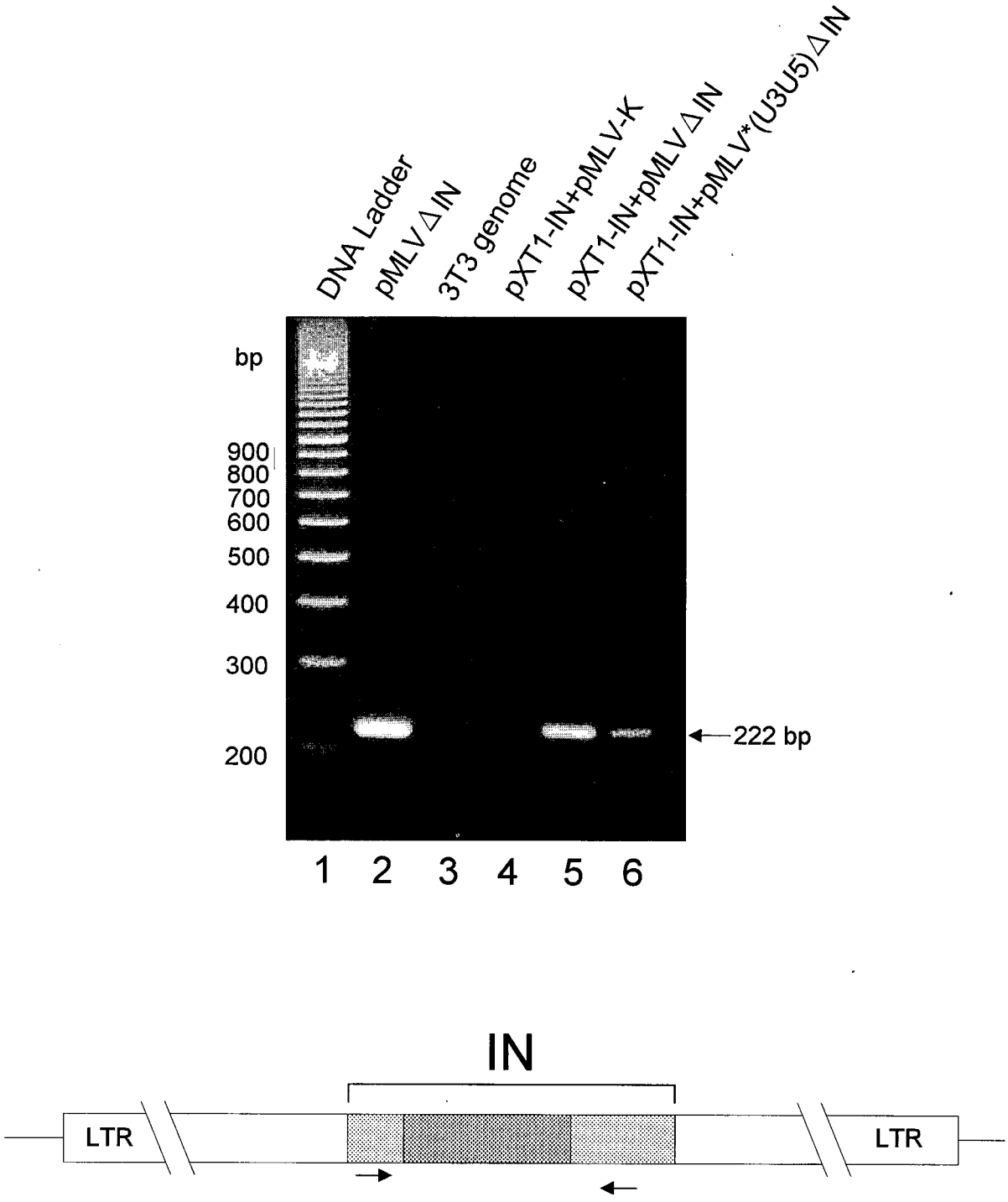


Fig. 8. PCR analysis of the genomic DNA extracted from NIH/3T3 cells transfected with vector pMLV Δ IN or of the genomic DNA extracted from HIV-1 IN expressing cells transfected with vectors pMLV-K, pMLV Δ IN, and pMLV*(U3U5) Δ IN using a primer set that could detect the portion of MuLV IN remaining after partial deletion. The binding positions of these primers are depicted in the diagram below the agarose gel used to resolve the generated PCR products. The annealing-extension step used in these PCR analyses was fixed at 70 °C for 15 sec only. Lane 1 was a DNA ladder; lane 2, PCR analysis of the genomic DNA extracted from the NIH/3T3 cells singly transfected with vector pMLV Δ IN; lane 3, PCR analysis on the genomic DNA extracted from NIH/3T3 cells; lane 4, PCR analysis of the genomic DNA extracted from the HIV-1 IN expressing cells transfected with vector pMLV-K; lanes 5 and 6 were PCR analyses of the genomic DNA extracted from the HIV-1 expressing cells transfected with vectors pMLV Δ IN and pMLV*(U3U5) Δ IN, respectively. Note that no PCR product was detected for the HIV-1 IN expressing cells transfected with vector pMLV-K (lane 4) for the short extension time used in the PCR analysis.

Infectious MuLV progeny particles were produced by cells containing MuLV genome with partially deleted MuLV IN and integrated by the expressed HIV-1 IN. The MuLV IN is approximately 14 KDa larger than the HIV-1 and avian sarcoma leukosis virus IN's (Johnson *et al.*, 1986; Jonsson *et al.*, 1996; Kulkosky *et al.*, 1992). This larger size of MuLV IN is accounted for by the difference in its N- and C- termini. The minimal domain for enzymatic activity for MuLV IN includes a complete DD(35)E region and most of the C-terminus (Jonsson *et al.*, 1996). The positions of *Hind* III and *Nde* I sites which we used to partially delete the MuLV IN were at amino acid residues 95 and 265, respectively. The *Hind* III was right in between the CC residues of the HHCC motif while the *Nde* I site was at Tyr265, which was 45 residues longer than the E residue of the DD(35)E domain (Jonsson *et al.*, 1996). Therefore, the deletion we made for MuLV IN was sufficient to remove the entire central catalytic domain of the protein. No virus particles were produced for the NIH/3T3 cells singly transfected with the MuLV IN partially deleted vector pMLV Δ IN.

Earlier *in vitro* integration studies revealed that the HIV-1 IN is not able to cleave the MuLV U5 oligonucleotide with high specificity (Vink *et al.*, 1991). In an *in vitro* integration reaction, substrate specificity required by the MuLV IN was not as stringent as that required by HIV-1 IN (Bushman and Craigie, 1990; Vink *et al.*, 1991; Vink *et al.*, 1990). Mutation of the highly conserved CA dinucleotides at the CJ to TA was tolerable by the MuLV IN but not by the HIV-1 IN (Bushman and Craigie, 1990; Vink *et al.*, 1991; Vink *et al.*, 1990). Compared with earlier *in vitro* integration studies (Bushman and Craigie, 1991; Chow and Brown, 1994; Chow and Brown, 1992; LaFemina *et al.*, 1991; Leavitt *et al.*, 1992; Vink *et al.*, 1991; Vink *et al.*, 1990), the changes we made for both the U3 and U5 att sequences might have been adequate for the HIV-1 IN since, after mutation, eight nucleotides from the CJ of the U3 att and nine nucleotides from the CJ of the U5 att sequences were exactly similar to those of the corresponding HIV-1 sequences. Due to the high rate of recombination during retrovirus replication, the stability of the mutated U3 and U5 att sequences were examined through DNA sequencing on the genomic DNA extracted from the infected cells. We found that these mutations were stable in the infection stage. The integration efficiency might have been enhanced if both pairs of att sequences on both the U3 and U5 regions were mutated. However, it was uncertain whether or not such a result would affect the reverse transcription. An earlier *in vivo* integration study using an infectious HIV-1 vector and an att site mutant with a deletion of ten nucleotides from the CJ produced an integration efficiency of 5% (Masuda *et al.*, 1995). This integration efficiency was increased to 40% if the only mutation made was to convert the highly conserved CA dinucleotides to TG (Masuda *et al.*, 1995). Using purified avian IN and oligonucleotide-plasmid as a donor-acceptor pair, several groups have shown that IN can perform the concerted integration reaction *in vitro* like that observed *in vivo* (Aiyar *et al.*, 1996; Donzella *et al.*, 1996; Vora *et al.*, 1994). However, the integration efficiency reported was only about 0.5% even both ends of the used donor substrate containing the WT viral sequences (Aiyar *et al.*, 1996). This integration efficiency could be enhanced about four-fold through the addition of a ubiquitous DNA-bending protein to the integration reaction (Aiyar *et al.*, 1996). Based on a comparison of the results of these *in vitro* and *in vivo* studies, it appears that substrate specificity is not so important when there is assistance from other proteins during the integration reaction. Several other studies also showed that substrate alterations could cause severe defects when analyzed as single-LTR donors but could cause mild defects when analyzed *in vivo* (Bushman and Craigie, 1990; Donzella *et al.*, 1993; Murphy *et al.*, 1993; Woerner *et al.*, 1992). It is also possible that conditions favoring the formation of a multimeric form of IN may boost the integration efficiency (Ellison *et al.*, 1995). It is generally believed that the active form of the retroviral IN protein is probably a multimer. This has been supported by kinetic data (Jones *et al.*, 1992), *in vitro* complementation experiments (Donzella *et al.*, 1996; Engelman *et al.*, 1993; van Gent *et al.*, 1993), and integration inhibition assay by a murine monoclonal antibody (Barsov *et al.*, 1996).

Production of virions from infected cells with integrated MuLV provirus genome by the expressed HIV-1 IN could persist for over 21 days. These cells were probably infected by defective MuLV particles containing functional HIV-1 IN rather than MuLV IN proteins since they were not selectable by G418. However, cells infected by virions produced from transfection of the HIV-1 IN expressing cells with the WT MuLV provirus vector were selectable by G418. This indicated that the drug resistant gene engineered on the pXT1-IN vector was transferred to the infected cell. Since there were WT att sequences on both the U3 and the U5 LTR of the vector, it is possible that the vector was integrated by the MuLV IN in the infected cells. Given that both the HIV-1 IN and MuLV IN were present in the same infected cells and that the pXT1-IN vector was integrated by the latter, the advantage of help from other viral proteins in the integration process was evident. Since the HIV-1 IN was expressed alone in the cells, no other help from viral proteins was possible for the protein. Recent genetic analysis on the HIV-1 IN has

shown that certain mutants of IN can interfere with a variety of steps in the early and late stages of the viral life cycle. For example, mutations in the zinc finger-like domain affect stages at or prior to initiation of reverse transcription (Masuda *et al.*, 1995) while a single H12N mutation can affect virus assembly and maturation (Engelman *et al.*, 1995). For MuLV IN, some N-terminal, core, and C-terminal domain mutations have produced lower levels of RT and IN (Roth, 1991; Roth *et al.*, 1990). In this experiment, we observed that to increase the integration efficiency for viral genome *in vivo* help from viral proteins was more important than that from cellular proteins.

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References

Aiyar, A., Ge, Z. and Leis, J. (1994) A specific orientation of RNA secondary structures required for initiation of reverse transcription. *J. Virol.*, **68:**611-618.

Aiyar, A., Hindmarsh, P., Skalka, A.M. and Leis, J. (1996) Concerted integration of linear retroviral DNA by the avian sarcoma virus integrase *in vitro*: dependence on both long terminal repeat termini. *J. Virol.*, **70:**3571-3580.

Barsov, E.V., Huber, W.E., Marcotrigiano, J., Clark, P.K., Clark, A.D., Arnold, E. and Hughes, S.H. (1996) Inhibition of human immunodeficiency virus type 1 integrase by the Fab fragment of a specific monoclonal antibody suggests that different multimerization states are required for different enzymatic functions. *J. Virol*, **70:**4484-4494.

Boulter, C.A. and Wagner, E.F. (1987) A universal retroviral vector for efficient constitutive expression of exogenous genes. *Nucleic Acids Res.*, **15:**7194-7194.

Burke, C.J., Sanyal, G., Bruner, M.W., Ryan, J.A., LaFemina, R.L., Robbins, H.L., Zeft, A.S., Middaugh, C.R. and Cordingley, M.G. (1992) Structural implications of sectroscopic characterization of a putative zinc-finger peptide from HIV-1 integrase. *J. Biol. Chem.*, **267:**9639-9644.

Bushman, F.D. and Craigie, R. (1990) Sequence requirements for integration of Moloney murine leukemia virus DNA *in vitro*. *J. Virol.*, **64:**5645-5648.

Bushman, F.D. and Craigie, R. (1991) Activities of human immunodeficiency virus (HIV) integration protein *in vitro*: specific cleavage and integration of HIV DNA. *Proc. Natl. Acad. Sci. USA*, **88:**1339-1343.

Bushman, F.D., Engelman, A., Palmer, I., Wingfield, P. and Craigie, R. (1993) Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. *Proc. Natl. Acad. Sci. USA*, **90:**3428-3432.

Bushman, F.D. and Wang, B. (1994) Rous sarcoma virus integrase protein: mapping functions for catalysis and substrate binding. *J. Virol.*, **68:**2215-2223.

Cannon, P.M., Wilson, W., Byles, E., Kingsman, S.M. and Kingsman, A.J. (1994) Human immunodeficiency virus type 1 integrase: effect on viral replication of mutations at highly conserved residues. *J. Virol.*, **68:**4768-4775.

Cheng, J.W., Cheng, C.C., Lyu, P.C., Chen, S.T. and Lin, T.H. (1996) Solution conformation of a peptide corresponding to residues 151-172 of HIV-1 integrase using NMR and CD spectroscopy. *Int. J. Peptide Protein Res.*, **47:**117-122.

Chow, S.A. and Brown, P.O. (1994) Substrate features important for recognition and catalysis by human immunodeficiency virus type 1 integrase identified by using novel DNA substrates. *J. Virol.*, **68:**3896-3907.

Chow, S.A., Vincent, K.A., Ellison, V. and Brown, P.O. (1992) Reversal of integration and DNA splicing mediated by integrase of human immunodeficiency virus. *Science*, **255:**723-726.

Cobrinik, D., Soskey, L. and Leis, L. (1988) A retroviral RNA secondary structure required for efficient initiation of reverse transcription. *J. Virol.*, **62:**3622-3630.

Collicelli, J. and Goff, S.P. (1988) Sequence and spacing requirements of a retrovirus integration site. *J. Mol. Biol.*, **199:**47-59.

Cone, R.D. and Mulligan, R.C. (1984) High efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. USA*, **81:**6349-6353.

Craigie, R., Fujiwara, T. and Bushman, F. (1990) The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell*, **62:**829-837.

Donzella, G.A., Jonsson, C.B. and Roth, M.J. (1993) Influence of substrate structure on disintegration activity of Moloney murine virus integrase. *J. Virol.*, **67:**7077-7087.

Donzella, G.A., Jonsson, C.B. and Roth, M.J. (1996) Coordinated disintegration reactions mediated by Moloney murine leukemia virus integrase. *J. Virol.*, **70:**3909-3921.

Drelich, M., Wilheim, R. and Mous, J. (1992) Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein *in vitro*. *Virol.*, **188:**459-468.

Dyda, F., Hickman, A.B., Jenkins, T.M., Engelman, A., Craigie, R. and Davies, D.R. (1994) Crystal structure of the catalytic domain of HIV integrase: similarity to other polynucleotidyl transferases. *Science*, **266:**1981-1986.

Ellison, V., Gerton, J., Vincent, K.A. and Brown, P.O. (1995) An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer. *J. Biol. Chem.*, **270:**3320-3326.

Engelman, A., Bushman, F.D. and Craigie, R. (1993) Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. *EMBO J.*, **12:**3269-3275.

Engelman, A. and Craigie, R. (1992) Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function *in vitro*. *J. Virol.*, **66:**6361-6369.

Engelman, A., Englund, G., Orenstein, J.M., Martin, M.A. and Craigie, R. (1995) Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J. Virol.*, **69:**2729-2736.

Engelman, A., Mizuuchi, K. and Craigie, R. (1991) HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell*, **67:**1211-1221.

Fujiwara, T. and Mizuuchi, K. (1988) Retroviral DNA integration: structure of an integration intermediate. *Cell*, **54:**497-504.

Johnson, M.S., McClure, M.A., Feng, F. D., Gray, J. and Doolittle, R.F. (1986) Computer analysis of retroviral pol genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. *Proc. Natl. Acad. Sci. USA*, **83:**7648-7652.

Jones, K.S., Coleman, J., Merkel, W.M., Laue, T.M. and Skalka, A.M. (1992) Retroviral integrase functions as a multimer and can turn over catalytically. *J. Biol. Chem.*, **267:**16037-16040.

Jonsson, C.B., Donzella, G.A., Gaucan, E., Smith, C.M. and Roth, M.J. (1996) Functional domains of Moloney murine leukemia virus integrase defined by mutation and complementation analysis. *J. Virol.*, **70:**4585-4597.

Jonsson, C.B. and Roth, M. J. (1993) Role of His-Cys finger of Moloney murine leukemia virus protein in integration and disintegration. *J. Virol.*, **67:**5562-5571.

Kalpana, G.V., Marmon, S., Wang, M., Crabtree, G. R. and Goff, S.P. (1994) Binding and stimulation of HIV-1 integrase by a human homology of yeast transcription factor SNF5. *Science*, **266:**2002-2006.

Katz, R.A., Merkel, G., Leis, J. and Skalka, A.M. (1990) The avian retroviral IN protein is both necessary and sufficient for integrative recombination *in vitro*. *Cell*, **63:**87-95.

Katzman, M., Katz, R.A., Skalka, A.M. and Leis, J. (1989) The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the *in vivo* sites of integration. *J. Virol.*, **63:**5319-5327.

Krogstad, P.A. and Champoux, J.J. (1990) Sequence specific binding of DNA by the Moloney murine leukemia virus integrase protein. *J. Virol.*, **64:**2796-2801.

Kulkosky, J., Jones, K.S., Katz, R.A., Mack, J.P.G. and Skalka, A.M. (1992) Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. *Mol. Cell Biol.*, **12:**2331-2338.

LaFemina, R.L., Callahan, P.L. and Cordingley, M.G. (1991) Substrate specificity of recombinant human immunodeficiency virus integrase protein. *J. Virol.*, **65:**5624-5630.

LaFemina, R.L., Schneider, C.L., Robbins, H.L., Callahan, P.L., LeGrow, K., Roth, E., Schleif, W.A. and Emini, E.A. (1992) Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. *J. Virol.*, **66:**7414-7419.

Leavitt, A.D., Rose, R.B. and Varmus, H.E. (1992) Both substrate and target oligonucleotide sequences affect *in vitro* integration mediated by human immunodeficiency virus type 1 integrase protein in *Saccharomyces cerevisiae*. *J. Virol.*, **66:**2359-2368.

Leavitt, A.D., Shiue, L. and Varmus, H.E. (1993) Site-directed mutagenesis of HIV-1 integrase demonstrates differential effects on integrase function *in vitro*. *J. Biol. Chem.*, **268:**2113-2119.

Levy, J.A. (1993) The Retroviridae, Vol. 2. Plenum Publishing, New York.

Lin, T.H. and Grandgenett, D.P. (1991) Retrovirus integrase: identification of a potential leucine zipper motif. *Protein Eng.*, **4:**435-441.

Lynch, C.M. and Miller, A.D. (1991) Production of high-titer helper virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. *J. Virol.*, **65:**3887-3890.

Masuda, T., Planelles, V., Krogstad, P. and Chen, I.S.Y. (1995) Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 att site: unusual phenotype of mutants in the zinc finger-like domain. *J. Virol.*, **69:**6687-6696.

McEuen, A.R., Edwards, B., Koepke, K.A., Ball, A.E., Jennings, B.A., Wolstenholme, A.J., Danson, M.J. and Hough, D.W. (1992) Zinc binding by retroviral integrase. *Biochem. Biophys. Res. Commun.*, **189:**813-818.

Miller, A.D. and Verma, I.M. (1984) Two base changes restore infectivity to a noninfectious molecular clone of Moloney murine leukemia virus (pMLV-1). *J. Virol.*, **49:**214-222.

Murphy, J.E., de Los Santos, T. and Goff, S.P. (1993) Mutational analysis of the sequences at the termini of the Moloney murine leukemia virus DNA required for integration. *Virology*, **195:**432-440.

Murphy, J.E. and Goff, S.P. (1989) Construction and analysis of deletion mutations in the U5 region of Moloney murine leukemia virus: effects on RNA packaging and reverse transcription. *J. Virol.*, **63:**319-327.

Perlak, F.J. (1990) Single step large scale site-directed *in vitro* mutagenesis using multiple oligonucleotides. *Nucleic Acids Res.*, **18:**7457-7458.

Puras Lutzke, R.A., Vink, C. and Plasterk, R.H.A. (1994) Characterization of the minimal DNA binding domain of the HIV integrase protein. *Nucleic Acids Res.*, **22:**4125-4131.

Roth, M.J. (1991) Mutational analysis of the carboxyl terminus of the Moloney murine leukemia virus integration protein. *J. Virol.*, **65:**2141-2145.

Roth, M.J., Schwartzberg, P.L. and Goff, S.P. (1989) Structure of the termini of DNA intermidiates in the integration of retroviral DNA dependence on IN function and terminal DNA sequence. *Cell*, **58:**47-54.

Roth, M.J., Schwartzberg, P.L., Tanese, N. and Goff, S.P. (1990) Analysis of mutations in the integration function of Moloney murine leukemia virus: effects on DNA binding and cutting. *J. Viro.*, **64:**4709-4717.

Schauer, M. and Billich, A. (1992) The N-terminal region of HIV-1 integrase is required for integration activity but not for DNA-binding. *Biochem. Biophys. Res. Commun.*, **185:**874-880.

Sherman, P.A. and Fyfe, J.A. (1990) Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. *Proc. Natl. Acad. Sci. USA*, **87:**5119-5123.

Shin, C.G., Taddeo, B., Haseltine, W.A. and Farnet, C.M. (1994) Genetic analysis of the human immunodeficiency virus type 1 integrase protein. *J. Virol.*, **68:**1633-1642.

Silver, J. and Keerikatte, V. (1989) Novel use of polymerase chain reaction to amplify cellular DNA adjacent to an integrated provirus. *J. Virol.*, **63:**1924-1928.

van Gent, D.C., Oude Groeneger, A.A.M. and Plasterk, R.H.A. (1992) Mutational analysis of the integrase protein of human immunodeficiency virus type 2. *Proc. Natl. Acad. Sci. USA*, **89:**9598-9602.

van Gent, D.C., Vink, C., Oude Groeneger, A.A.M. and Plasterk, R.H.A. (1993) Complementation between HIV integrase proteins mutated in different domains. *EMBO J.*, **12:**3261-3267.

Vincent, K.A., Ellison, V., Chow, S.A. and Brown, P.O. (1993) Characterization of human immunodeficiency virus type 1 integrase expressed in *Escherichia coli* and analysis of variants with amino-terminal mutations. *J. Virol.*, **67:**425-437.

Vink, C., Oude Groeneger, A.A.M. and Plasterk, R.H.A. (1993) Identification of the catalytic and DNA binding region of the human immunodeficiency virus type 1 protein. *Nucleic Acids Res.*, **21:**1419-1425.

Vink, C., van Gent, D.C., Elgersma, Y. and Plasterk, R.H.A. (1991) Human immunodeficiency virus integrase protein requires a subterminal position of its viral DNA recognition sequence for efficient cleavage. *J. Virol.*, **65:**4636-4644.

Vink, C., van Gent, D.C. and Plasterk, R.H.A. (1990) Integration of human immunodeficiency virus types 1 and 2 DNA *in vitro* by cytoplasmic extracts of Moloney murine leukemia virus-infected mouse NIH3T3 cells. *J. Virol.*, **64:**5219-5222.

Vink, C., Yeheskiely, E., van der Marel, G.A., van Boom, J.H. and

Plasterk, R.H.A. (1991) Site-specific hydrolysis and alcoholysis of human immunodeficiency virus DNA termini mediated by the viral integrase protein. *Nucleic Acids Res.*, **19:**6691-6698.

Vora, A.C., Fitzgerald, M.L. and Grandgenett, D.P. (1990) Removal of 3'-OH-terminal nucleotides from blunt-ended long terminal repeat termini by the avian retrovirus integration protein. *J. Virol.*, **64:**5656-5659.

Vora, A.C., McCord, M., Fitzgerald, M.L., Inman, R.B. and Grandgenett, D.P. (1994) Efficient concerted integration of retrovirus-like DNA *in vitro* by avian myeloblastosis virus integrase. *Nucleic Acids Res.*, **224:**4454-4461.

Wang, C.Y., Yang, C.F., Lai, M.C., Lee, Y.H., Lee, T.L. and Lin, T.H. (1994) Molecular dynamics simulation of a leucine zipper motif predicted for the integrase of human immunodeficiency virus type 1. *Biopolymers*, **34:**1027-1036.

Woerner, A.M., Klutch, M., Levin, J.G. and Marcus-Sekura, C.J. (1992) Localization of DNA binding activity of HIV-1 integrase to the C-terminal half of the protein. *AIDS Res. Hum. Retroviruses*, **8:**297-304.

Woerner, A.M. and Marcus-Sekura, C.J. (1993) Characterization of a DNA binding domain in the C-terminus of HIV-1 integrase by deletion mutagenesis. *Nucleic Acids Res.*, 21:3507-3511.

鼠類白血病毒的基因組可被單獨表現在細胞中的愛滋病毒的核酸銜接酵素所銜接

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摘　要

爲研究反轉錄病毒的核酸銜接過程，我們將愛滋病毒(HIV-1)的核酸銜接酵素(HIV-1 IN)單獨的表現在細胞中，並使用攜帶著完整鼠類的白血病毒(Mo-MuLV)基因組載體做爲其受質，以設計了一個活體內的核酸銜接分析方法。取自載體pINSD的HIV-1 IN基因先被選殖入載體pXT1，以產生能表現HIV-1 IN的載體pXT1-IN。載體pMLV-K攜有完整的Mo-MuLV基因組，而且具感染性。在此載體原Mo-MuLV IN能作用上去的circle junction上的U3及U5 attachment (att)序列，也被以定點突變的方法改變成HIV-1 IN，所能作用者以產生載體pMLV*(U3U5)。然後把在載體pMLV-K及pMLV*(U3U5)上的Mo-MuLV IN的序列做部份切除，以產生載體pMLV Δ IN及pMLV*(U3U5) Δ IN。在用這些載體分別轉化感染NIH/3T3細胞之後，爲偵測Mo-MuLV的基因組是否有被HIV-1 IN所銜接，我們使用一種非放射性的反轉錄酵素(RT)活性分析法，以察看是否有病毒粒子自細胞中被釋出。我們發現被載體pMLV Δ IN所單獨感染的細胞並無病毒粒子的釋出。不過分別被載體pMLV Δ IN或pMLV*(U3U5) Δ IN 所轉化感染，且能表現HIV-1 IN的細胞卻有病毒粒子的釋出，因爲有明顯的RT活性被測到。此結果顯示單獨被表現在細胞中的HIV-1 IN，可將完整的Mo-MuLV基因組銜接上細胞的染色體。此外，我們將含有被銜接上的Mo-MuLV基因組的細胞基因組抽出，並以被部份切除的Mo-MuLV IN序列做爲目標序列，以使用多鏈聚合反應，進一步證明HIV-1 IN可單獨將Mo-MuLV的基因組銜接上細胞的染色體。