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Expression of Human Immunodeficiency Virus Type 1 Reverse Transcriptase in *trans* during Virion Release and after Infection

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The normal reverse transcription of retroviral RNA is a complex process which depends on the orchestration of several steps throughout the virus life cycle. During the assembly of retroviruses, reverse transcriptase (RT) is directed into the virion as a component of the Gag-Pol polyprotein. In the maturation of the Gag-Pol polyprotein of human immunodeficiency virus type 1 (HIV-1), cleavage by the viral protease occurs during viral budding. After infection, reverse transcription of viral RNA into double-stranded DNA is completed in the cytoplasm of the infected cell. In this study, the processing and reverse transcription of HIV-1 have been examined by separate expression of mature HIV-1 RT and proviral molecules bearing RT mutations. The effects of RT expression in *trans* during virion release and after viral entry were investigated. Constitutive expression of HIV-1 RT was established in CD4- and non-CD4-expressing cells via the coexpression of its individual subunits, and three HIV-1 RT mutant constructs were generated. The results indicate that a bona fide RT *trans* complementation does not occur during virion release or after infection. However, after infection of an RT-expressing cell with a high titer RT-defective virus, intracellular reverse transcription can be detected.

The key enzyme in the replication of human immunodeficiency virus type 1 (HIV-1) is reverse transcriptase (RT). The RT region of the *pol* gene encodes a 66-kDa subunit (p66), and an equimolar amount is cleaved into a 51-kDa subunit (p51) by HIV-1 protease (PR) to form the functional heterodimer (8, 10, 18). The *pol* gene is synthesized as a component of the Gag-Pol polyprotein precursor ($Pr160^{gag\text{-}pol}$) that is incorporated into the virion via its association with the Gag polyprotein precursor ($Pr55^{gag}$) (25, 32). Processing of $Pr160^{gag\text{-}pol}$ by PR is believed to occur during the virus budding, which is presumably initiated at the membrane of the infected cell (15).

The reverse transcription of the viral RNA involves a series of events at two stages of the virus life cycle. During the early phase, after entry into the cytoplasm, the reverse transcription of viral RNA is initiated and proceeds to completion within the tightly associated ribonucleocapsid environment. This is followed by the integration of double-stranded viral DNA into the host genome. However, partial reverse transcription of the viral RNA may occur in the virion before infection (20, 39, 40). During the late phase, the necessary components for the reverse transcription process, including two copies of plus-strand viral RNA, RT, $tRNA_3^{Lys}$, and nucleocapsid proteins, are packaged and processed in the virus (for reviews, see references 12 and 36).

Different mutations in HIV-1 can be complemented by expression of the functional viral components in *trans*. The *trans* complementation of some of the viral components, such as gp160 and Vpr, can be accomplished by their expression during virion assembly (6, 24, 26). For other viral components, including Tat and Rev, *trans* complementation can be achieved at both stages of the virus life cycle (2, 5). Furthermore, some mutant viruses can *trans* complement by coinfecting the same cell (19, 38).

In the present study, the effect of independent expression of RT on RT mutant viruses has been analyzed at two stages of virus life cycle. Previously, we demonstrated constitutive cytoplasmic expression of active HIV-1 RT in HT-1080 cells by coexpressing the individual subunits (4). Constitutive expression of HIV-1 RT heterodimer has now been established in the HeLa-CD4-LTR/β-galactosidase (HCB) cell line. In addition, three different HIV-1 RT mutant constructs were generated. Our results indicate that RT expressed in *trans* is not packaged during the virion release; however, intracellular reverse transcription can be detected at a low level after infection of an RT-expressing cell, in the absence of productive infection.

MATERIALS AND METHODS

Cell culture. The HT-1080 cell line (27), the pSV51/pCMV66 stable cell line (4), and HeLa-derived cell lines were all maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah)–2 mM L-glutamine–penicillin (50 U/ml)–streptomycin (50 μg/ml) (GIBCO BRL, Gaithersburg, Md.). The HeLa-CD4-LTR/β-Galactosidase cell line (HCB) was obtained from M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. For HCB cells and the pSV51/pCMV66 stable cell line, medium was supplemented with active Geneticin (G418; 200 μg/ml) (GIBCO-BRL), and for HCB cells, hygromycin B (100 μg/ml) (Sigma, St. Louis, Mo.) was included. Establishment of stable RT expression in HCB cells (HCBRT cells) was achieved essentially as described previously (4). Briefly, HCB cells were transfected with linearized pCMV66 and pSV51 constructs along with a pPGKpurobpA construct (a gift from Allan Bradley) and then selected for puromycin resistance. For selection and maintenance of HCBRT cells, 2 μg of puromycin (Sigma) per ml was additionally included in the medium. For multinuclear activation of the β-galactosidase indicator (MAGI) assaying, low (2 to 5 ng of p24)- or high-titer (30 to 50 ng of p24) virus was used to infect HCB or HCBRT cells. Infections, staining for β-galactosidase gene expression, and blue cell counts were performed as described elsewhere (16). The blue cell counts are averages of three separate measurements.

Plasmid constructions. Construction of pCMV66 and pSV51 has been described previously (4). These constructs express p66 and p51, respectively. Different segments of *pol* gene of pNL4-3 molecular clone (1) were amplified by PCR with primer pairs R646 (5′-GACTCCAATGATGGCCATTCCTTTGGATGGGTTATG-3′)/R584 (5′-CAGGCTAAGACTGGCCATCTTCCTGCTAATTTTAAG-3′) and R585 (5′-GACTCCAATGATGGCCAGCACACAAAGGAATTGGAG-3′)/R584 (note: each primer has an *Msc*I site which is underlined) and were cloned in place of the *Msc*I fragment of pNL4-3 (2621-4553) to generate the RT603 and RT1536 constructs, respectively. By this strategy, 603- and 1,536-bp in-frame deletions in the RT region of pNL4-3 were generated.

To generate the YMAA construct, a megaprimer PCR strategy was utilized (30). Initially, primers R330 (5′-GTTAAACAATGGCCATTGACAG-3′) and R622 (5′-CCTACATACAAAGCAGCCATGTATTGATAGATGAC-3′) were used to amplify a segment of the pNL4-3 molecular clone with R622 containing

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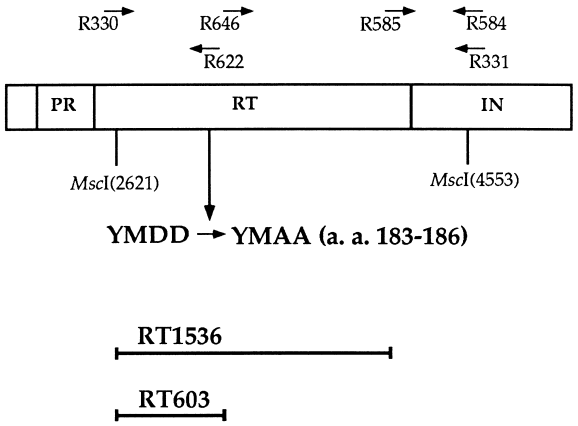

FIG. 1. Schematic representation of the pNL4-3 *pol* gene. Arrows, oligonucleotides used for the generation of the mutant constructs; bars, deletion regions in RT1536 and RT603. The amino acid (a.a.) position of the mutated YMDD→YMAA motif of RT is indicated.

the substitution bases. The product of this PCR was used with primer R331 (5′-GTTTTTACTGGCCATCTTCC-3′) to amplify the *Msc*I segment of pNL4-3. This mutated fragment was cloned in place of the wild-type *Msc*I fragment of pNL4-3. The sequences of all of the constructs were confirmed by the *Bst* solid-phase sequencing approach (unpublished data).

Virus production. Various constructs were transfected into HT-1080 cells or the pSV51/pCMV66 stable cell line by electroporation as described previously (4). At 24 h posttransfection, the cells were washed three times with phosphate-buffered saline and new medium was added. At 48 h after transfection, supernatants were collected, centrifuged at 300 × *g* for 5 min, and filtered (0.45-μm-pore-size filters). For low-titer experiments, various fractions of these supernatants were used for infection of HCB or HCBRT cells. To generate a higher titer of virus, virions were pelleted directly (85,000 × *g*; 4°C; 1 h) or by centrifugation through a 20% sucrose cushion (85,000 × *g*; 4°C; 4 h). The viral pellets were lysed for Western blot (immunoblot) analysis and RT activity assays (3). The p24 concentrations of viral supernatants, viral pellets, or viral lysates were measured by Coulter HIV-1 p24 antigen assays (Coulter Corp., Miami, Fla.) according to the manufacturer's specifications.

Detection of 1-LTR and 2-LTR circle junctions. For detection of 1-LTR and 2-LTR circle junctions, virus stocks were first DNase treated. Approximately 8×10^4 HCB or HCBRT cells were infected with a low viral titer (2 to 5 ng of p24) or with a high titer (20 to 50 ng of p24) as described elsewhere (16). Nucleic acid isolation was performed at the indicated times as described elsewhere (11). For the PCRs, 200 ng of *Dpn*I-digested nucleic acid was used in a nested PCR strategy as previously described (3). Briefly, primers R685 and R687 were used in the first PCR for 28 cycles. For the second round of PCR, 1 μl of the first reaction mixture was amplified (30 cycles) with the nested primers R686/R684 for 1-LTR junctions and R628/R630 for 2-LTR junctions.

Immunoblotting. Western blot analyses of total viral proteins, gp120, IN, and RT were performed as previously described (3). The immunoglobulin G (IgG) antibody (Ab) purified from pooled plasma of HIV-1-seropositive donors and HIV-1 IIIB gp120 antiserum were obtained, respectively, from A. Prince and M. Phelan through the AIDS Research and Reference Reagent Program. HIV-1 SF2 gp120 and capsid protein (CA) were obtained from K. Steimer, Chiron Corp., through the AIDS Research and Reference Reagent Program. For detection of intracellular RT, total viral protein, and gp120, alkaline phosphatase-conjugated anti-mouse, anti-human, and anti-sheep IgGs (Sigma), respectively, were used. For detection of virion-associated RT and integrase (IN), horseradish peroxidase-linked anti-mouse Ab was utilized (ECL TM Western blotting; Amersham).

RESULTS

Effects of RT mutations on Pr160$^{gag\text{-}pol}$. Three different HIV-1 RT mutant constructs were generated in a pNL4-3 infectious molecular clone background, as schematically represented in Fig. 1. The RT1536 and RT603 constructs, respectively, have 1536- and 603-bp in-frame deletions in the RT region of the *pol* gene. In the YMAA construct, the wild-type aspartic acid residues in the conserved YMDD motif (17, 21) were replaced with two alanine residues.

Initially, the effect of mutations in RT on Pr160$^{gag\text{-}pol}$ stability and/or packaging was investigated. Mutant and wild-type constructs were individually transfected into HT-1080 cells, and the released viruses were harvested by ultracentrifugation. As a negative control, supernatant from HT-1080 cells transfected with the pCMVβ construct (22) was processed in a similar fashion. Viral pellets were lysed and normalized to p24 levels. Compared with the wild-type virus, none of the RT mutant viruses exhibited any RT activity (data not shown). Western blot profiles of total viral proteins, RT, IN, and gp120 are presented in Fig. 2. Compared with pNL4-3, YMAA exhibited normal Pr160$^{gag\text{-}pol}$ packaging and processing, as determined by the level of virion-associated RT and IN. The Pr160$^{gag\text{-}pol}$ stability and/or packaging of RT1536 and RT603, however, was perturbed, as deduced from severe reduction in virion-associated IN (Fig. 2B). No RT product could be detected for RT1536 and RT603 because of the presence of large in-frame RT deletions in these mutants (Fig. 2C). A decrease in stability and/or packaging of Pr160$^{gag\text{-}pol}$ can lead to a similar decrease in virion-associated PR; therefore, Pr55^{gag} and Pr160$^{gag\text{-}pol}$ might not be completely processed. Since viral lysates were normalized to p24 levels, this could result in an artificial increase in gp120 (Fig. 2D) and other viral proteins (Fig. 2A), as observed for the RT1536 and RT603 mutations.

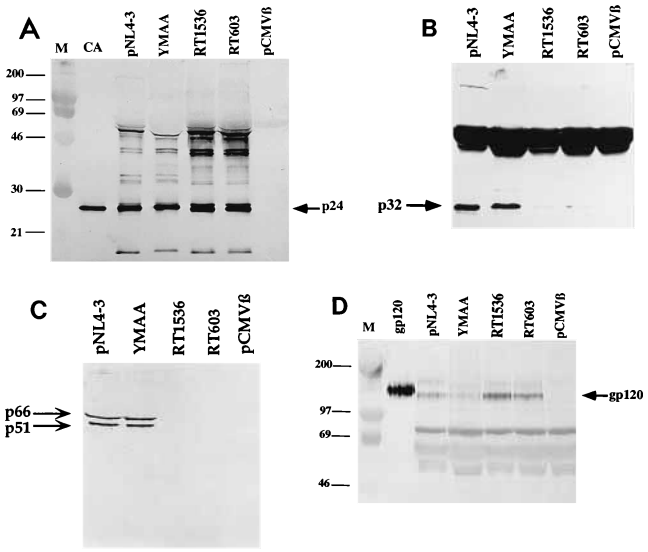

FIG. 2. Western blot profile of viral lysates. Approximately 12 ng of p24 equalized viral lysate was loaded in each lane. (A) Total viral proteins detected by IgG Ab purified from pooled plasma of HIV-1-seropositive donors; (B) IN protein (p32) detected by an anti-HIV IIIB IN IgG1 monoclonal Ab (Intracel, Cambridge, Mass.); (C) RT subunits detected by an anti-HIV IIIB RT IgG1 monoclonal Ab (Intracel); (D) gp120 detected by a sheep anti-HIV IIIB gp120 polyclonal Ab.

We also tested whether the insertion of an exogenous gene, with approximately the size of RT, could restore Gag-Pol packaging and processing of RT deletion mutants to normal levels. A cDNA encoding a luciferase protein (7) was cloned in place of RT, in the same frame as PR and IN. After transfection of this construct into HT-1080 cells, virus production was drastically reduced and Gag-Pol incorporation into virion was almost completely abolished. However, luciferase activity could be detected in the cell extract, suggesting the synthesis of Gag-Pol polyprotein but not its incorporation into the virion (data not shown).

RT expression in *trans* during virion release. Normally, RT is directed to the virus as a component of Pr160$^{gag\text{-}pol}$. To analyze the effect of RT expression in *trans* during the virion

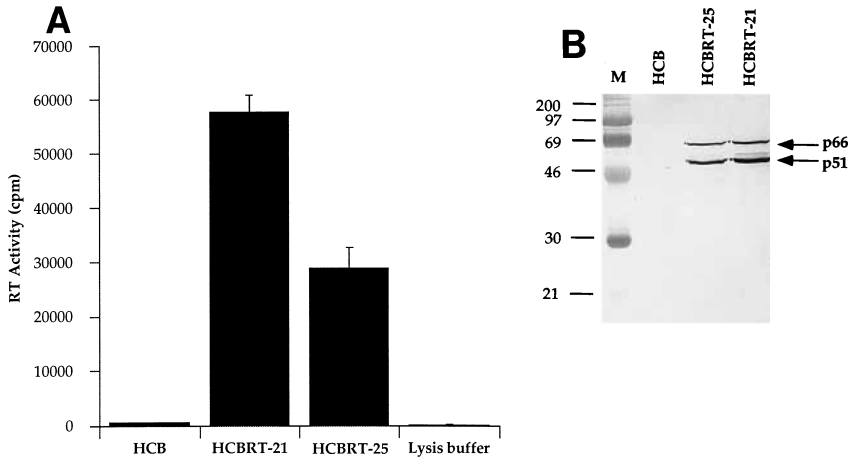

FIG. 3. Analysis of RT production in HCBRT stable cell lines. (A) RT activity assay of cell lysates. Each bar represents the average of three separate measurements. RT was assayed as described elsewhere (4). Approximately 6 μg of total cellular protein was analyzed from each cell extract in each measurement. (B) Western blot profile of cell lysates. Approximately 14 μg of total cellular protein per lane was loaded. M, molecular mass markers in kilodaltons. The total protein concentrations of the cell lysates were measured with a bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.).

release, the pSV51/pCMV66 cell line (4), which constitutively expresses active HIV-1 RT heterodimer, was separately transfected with the wild-type, RT603, RT1536, and pCMVβ constructs. At 48 h posttransfection, supernatants were harvested and pelleted by ultracentrifugation. RT activity assays and Western blot analysis of lysed pellets showed similar levels of RT released into the supernatant of cells transfected with RT1536, RT603, and pCMVβ (data not shown). These results suggest that when RT is expressed in *trans*, it cannot be packaged into the virus by its association with other viral components.

RT expression in *trans* after viral entry. To determine the effect of RT expression in *trans* after infection, the HCB cell line was chosen to establish constitutive HIV-1 RT expression. This HeLa-derived CD4-expressing cell line has a single integrated copy of the β-galactosidase gene under the control of a truncated HIV-1 LTR (16). Several stable cell lines were isolated (HCBRT cells), and RT expression was analyzed by immunoblotting and RT activity assays. The two cell lines indicated in Fig. 3 each produce high levels of RT. After infection of HCBRT cells with RT mutant viruses, two different assays were used for intracellular reverse transcription analysis.

The first test was the MAGI assay developed by Kimpton and Emerman (16). In HCB and HCBRT cells, expression of the β-galactosidase gene is contingent on the expression of HIV-1 Tat *trans*-activator protein. Reverse transcription of the viral RNA into double-stranded viral DNA is a necessary requirement for Tat expression. After infection of HCB or HCBRT cells with RT mutant viruses, β-galactosidase gene expression could not be detected above the background level at either a low or a high viral titer (Table 1). After infection with pNL4-3, HCBRT-21 and HCBRT-25 cells exhibited a two- to threefold increase in blue cell counts compared with HCB cells (Table 1). However, two other HCBRT clones (HCBRT-4 and HCBRT-8) did not show this increase (data not shown). This increase could represent an enhancement of the efficiency of reverse transcription of the wild-type virus by RT expression in *trans*, although this effect may also be due to factors specific to clonal selection of these cells.

The second test for possible *trans* activity was an assay for intracellular reverse transcription via the detection of 1-LTR and 2-LTR circular viral forms by PCR. The simple covalently closed 1-LTR and 2-LTR circular forms are believed to be the products of recombination and ligation of LTRs flanking the linear viral DNA, respectively (9, 31). HCB and HCBRT cells were infected with different titers of wild-type and RT mutant viruses. Since RT1536 and RT603 exhibited the same Gag-Pol packaging and processing, only RT603 and YMAA were further examined. At 24 h postinfection, nucleic acid was isolated and subjected to the nested PCR strategy (Fig. 4). Both 1-LTR and 2-LTR circle junctions could be detected for pNL4-3 at low and high titers of virus after infection of either of the cell lines. Neither 1-LTR nor 2-LTR products could be detected for RT mutant viruses at a low virus titer (data not shown). However, the 1-LTR and 2-LTR circle junctions could be detected after infection of HCBRT cells with RT mutant viruses at a higher viral titer (Fig. 4B and C). The 1-LTR junction was detected more frequently than the 2-LTR junction. In some of the infection experiments, both 1-LTR and 2-LTR junctions could not be detected, pointing to the low level of their formation (Fig. 4). However, in all control samples, neither 1-LTR nor 2-LTR products could be detected in HCB cells infected with the same preparation of RT mutant virus. Since the 1-LTR junction can theoretically be generated by PCR recombination, it is not known whether the amplifiable 1-LTR junctions represent full circular, full linear, or partial LTR-containing reverse-transcribed viral DNA. However, rare detection of 2-LTR junctions suggests the presence of some full circular molecules.

TABLE 1. Detection of β-galactosidase gene expression by the MAGI assay[a]

Virus	No. of blue cells		
	HCB	HCBRT-21	HCBRT-25
pNL4-3	370 ± 46	875 ± 53	890 ± 38
RT603	3 ± 1	2 ± 1	3 ± 2
RT1536	2 ± 1	3 ± 1	6 ± 2
YMAA	3 ± 2	2 ± 1	3 ± 1
Mock	1 ± 0	1 ± 0	2 ± 1

[a] For each cell line, approximately 8×10^4 cells were infected with equal p24 amounts of each RT mutant virus (40 ng). For pNL4-3, a lower virus titer was used (2.5 ng).

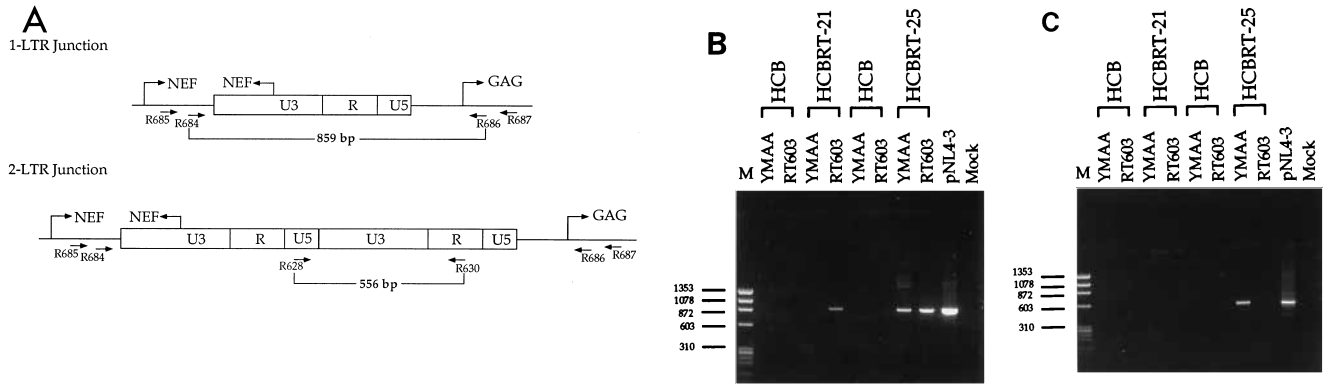

FIG. 4. Detection of 1-LTR and 2-LTR circle junctions by nested PCR. (A) Schematic representation of the junctions and the relative positions of the primers. (B and C) Ethidium bromide-stained agarose gels of 1-LTR junctions (895 bp) and 2-LTR junctions (556 bp), respectively. The pNL4-3 lanes indicate PCR from HCB cells infected with the wild-type virus. The mock lanes represent PCR from HCBRT-25 cells that are mock-infected. The viruses and the cell lines used for infection are indicated for the rest of the lanes. M, ϕX174 RF DNA-*Hae*III fragments.

To compare the efficiency of intracellular reverse transcription by RT expressed in *trans* with that of a similar process in the wild-type virus, HCB cells were infected with serial dilutions of pNL4-3. Twenty-four hours postinfection, 1-LTR and 2-LTR junctions were determined by PCR (Table 2). Separately, 48 h postinfection Tat expression was analyzed by the MAGI assay (Table 2). Compared with the wild-type virus, 1-LTR junction was formed at an approximately 1,000-fold-lower efficiency for RT-defective viruses.

DISCUSSION

In this study, the effects of HIV-1 RT expression in *trans* were examined at two stages of the virus life cycle: during packaging and release of the virus and after infection. RT, expressed in *trans*, is not packaged into RT-minus virus during its assembly and release. This result was expected, since RT is normally targeted into virions as a part of $Pr160^{gag\text{-}pol}$. However, upon infection of RT-expressing cells with RT mutant virus, intracellular reverse transcription occurs, albeit at a very low frequency, as demonstrated by the detection of 1-LTR and 2-LTR circle junctions by a nested-PCR strategy. Whether intracellular reverse transcription is an indication of the accessibility of the nucleocapsid microenvironment to the RT expressed in *trans* or whether cytoplasmic RT is reverse transcribing viral RNA which has leaked from this microenvironment has not been resolved.

TABLE 2. Detection of 1-LTR and 2-LTR junctions at various titers of pNL4-3[a]

Amt of p24 (ng)	No. of blue cells	Presence or absence	
		1-LTR junction	2-LTR junction
5	1,075 ± 86	+	+
0.5	114 ± 1	+	+
0.1	27 ± 2	+	−
0.05	11 ± 1	+	−
0.01	3 ± 2	−	−
0.005	3 ± 1	−	−
Mock infection	2 ± 1	−	−

[a] Approximately 8×10^4 HCB cells were infected with a serially diluted pNL4-3. Since approximately 40 to 50 ng of RT mutant virus was required for detection of the 1-LTR junction upon infection of HCBRT cells (see Fig. 4B) compared with the wild-type virus, the 1-LTR junction is formed at an approximately 1,000-fold-lower efficiency for the RT mutant virus.

During release of the virus, RT as a component of $Pr160^{gag\text{-}pol}$ can influence the polyprotein conformation. Large in-frame deletions in the RT region resulted in reduced stability and/or packaging of $Pr160^{gag\text{-}pol}$, as determined by a decrease in the level of virion-associated IN. Furthermore, the replacement of a luciferase cDNA for RT in-frame with PR and IN severely reduced virion release and Gag-Pol polyprotein stability and/or packaging. Since $Pr160^{gag\text{-}pol}$ is believed to be packaged into virions via its association with $Pr55^{gag}$ (25, 32), conformational changes in $Pr160^{gag\text{-}pol}$ can lead to its reduced packaging. In addition, PR and IN are targeted to the packaging site as parts of the $Pr160^{gag\text{-}pol}$. Therefore, viral maturation and viral DNA integration can also be disturbed by $Pr160^{gag\text{-}pol}$ conformational changes.

RT expression was established in an HCB cell line background to indirectly use Tat expression as an assay for detection of RT *trans* complementation. By this assay, Tat expression could not be detected either at a low or at a high viral titer. Viral gene expression, including Tat, is affected by the viral DNA integration (3, 29, 33, 37). In the absence of integration, Tat expression is reduced (3, 37). Absence or reduced integration of reverse-transcribed viral genome may partly account for the inability to detect Tat expression. If intracellular reverse transcription by RT in *trans* occurs after viral RNA leakage from the capsid/nucleocapsid microenvironment, viral DNA integration and gene expression might be abolished. In addition, our previous study indicated that Tat expression is severely affected by the level of virion associated RT (3). The concentration of RT in HCBRT cells might not be sufficient for efficient reverse transcription of the viral RNA and hence Tat synthesis.

Several factors, including structural constraints of the reverse transcription complex, timing of initiation of reverse transcription process, and RT-dependent packaging of cellular components, might contribute to the inefficiency of intracellular reverse transcription by RT expressed in *trans*. Other components of the reverse transcription complex probably provide a structural barrier to RT expression in *trans* that could limit RT *trans* complementation after infection. For YMAA mutant virus, nonfunctional RT is believed to be associated with the viral RNA (21), and hence it might also hinder the accessibility of functional RT. The timing of initiation of reverse transcription may also influence the efficiency and completion of this

process. Partial intravirion HIV-1 DNA has been detected in virions purified from peripheral blood plasma of infected individuals and virions purified from cell cultures (39). Furthermore, the presence of an intravirion RNA/DNA heteroduplex may be normally required to enhance RNA stability and increase the efficiency of the reverse transcription process (20, 39, 40). Finally, incorporation of $tRNA_3^{Lys}$ into the virion during its assembly may depend on the presence of RT as a component of $Pr160^{gag\text{-}pol}$ (13, 23). Since $tRNA_3^{Lys}$ is believed to be the primer for initiation of the reverse transcription process (14, 28), the possible perturbation in its packaging in RT mutant viruses may reduce the efficiency of the reverse transcription process.

Finally, our study has implications for modeling the mechanisms of retrotransposition of other LTR-containing elements by expression of RT in *trans*. The machinery for replication of retroviruses and that for intracellular retrotransposition of LTR-containing elements share similar structural and functional features (for a review, see reference 35). However, retroviruses can be transmitted horizontally via infection of a cell with the released viral particles. Intracellular transposition of defective retroviruses lacking *gag*, *pol*, and *env* genes can be accomplished by expression of *gag-pol* gene in *trans* (34). Our system suggests that expression of RT in *trans* might be sufficient for reverse transcription of LTR-containing elements, assuming that the Gag components are provided by the retrotransposon itself. The low level of detectability of intracellular reverse transcription is consistent with the low frequency of the retrotransposition process.

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