# Increasing Transduction Efficiency of Recombinant Murine Retrovirus Vectors by Initiation of Endogenous Reverse Transcription: Potential Utility for Genetic Therapies

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**Reverse transcription of retroviral genomic RNA in a target cell is influenced by cellular factors, including the concentration of deoxyribonucleoside triphosphates (dNTPs). In addition, recent data have demonstrated that reverse transcription can be driven within human immunodeficiency virus type 1 virions, prior to infection of a cell, by increasing extracellular concentrations of dNTPs. In attempts to increase the transduction efficiency of recombinant murine leukemia virus vectors, endogenous reverse transcription was initiated within cell-free, recombinant murine leukemia virus virions in the presence of relatively high concentrations of dNTPs. As a result, the expression of transduced genes via these retroviral vectors was increased approximately 10-fold by treatment of virions with dNTPs. Combined with our previous data, these observations suggest that virion-associated DNA synthesis can occur in diverse groups of retroviruses and positively alter retroviral infectivity. As such, these manipulations may be useful for increasing the efficiency of retrovirus-mediated gene delivery.**

To complete a retroviral life cycle, the viral genomic RNA carried by retroviral particles is reverse transcribed into viral DNA, which is integrated into the host chromosomal DNA. The integrated proviral DNA then behaves as a residual gene in the host cell's chromosome; i.e., DNA duplication during cell division and transcription of mRNA take place. On the basis of these characteristics, retroviral vectors have been widely used to deliver foreign genes into target cells (16, 22, 23). However, several potential problems for this delivery system remain. One is the efficiency of reverse transcription in certain target cells. Synthesis of full-length retroviral DNA is not detected in quiescent cells, partly because of the extremely low deoxyribonucleoside triphosphate (dNTP) concentrations in the quiescent cells, which are not sufficient to support efficient reverse transcription (12, 21, 24, 34). The compartmentalization of the dNTPs in certain proliferating cells may also limit available dNTPs for viral DNA synthesis (17, 18, 26).

Endogenous reverse transcription in retroviruses is traditionally considered a somewhat artificial process, which only mimics the reverse transcription occurring in the cytoplasm of target cells. Virion envelope permeabilization with reagents, such as nonionic detergents or melittin (a bee venom toxin), is routinely used for completion of the endogenous reverse transcription reaction (5, 13, 27). However, a certain level of reverse transcription can take place without detergent treatment of virions. This phenomenon was even demonstrated in the very early days of retroviral study (4, 30), and in recent years this process was detected in studies of human immunodeficiency virus type 1 virions (6, 10, 20, 31, 33, 36). Some authors attributed this phenomenon to possible damage of the viral envelope during the process of virion purification or to freezing and thawing (6, 10). However, we examined this phenom-

enon by different methods, including initiation of endogenous reverse transcription with fresh virion-containing supernatants of infected cells, prior to any isolation of viruses, and demonstrated that this process is not dependent upon artificial permeabilization of the virion envelope (reference 36 and unpublished data). Further, human immunodeficiency virus type 1 virions treated with concentrated dNTPs initiate additional intravirion DNA synthesis and have been shown to then possess increased infectivity, compared with untreated virions (36). On the basis of these observations, we hypothesized that the transduction efficiency of recombinant murine leukemia virus (MLV)-derived vectors may be augmented by initiating this endogenous reverse transcription prior to infection of target cells.

To demonstrate endogenous reverse transcription in recombinant MLV, a retroviral vector system similar to the one described previously (11) was utilized. Briefly, $10^6$ cells of the PA317 line, the packaging cell line which supplies the viral structural proteins (22), were plated onto 100-mm-diameter plates overnight. These cells were subsequently transfected with 50 μg of a plasmid containing the chloramphenicol acetyltransferase (CAT) gene driven from the MLV long terminal repeat in an MLV backbone, pLXSN-CAT (11, 23) (Fig. 1), by using the calcium phosphate precipitation system, as described previously (15). After 16 h, the supernatant was removed, the cells were washed with phosphate-buffered saline (PBS), and fresh Dulbecco's modified Eagle's medium plus 10% fetal calf serum was added. At 48 h posttransfection, the cell debris was eliminated by centrifugation at $300 \times g$ for 10 min and subsequently at $5,000 \times g$ for 10 min, and the supernatant was collected. The virions were then centrifuged onto a 20% sucrose cushion at $60,000 \times g$ for 40 min in a Ti 50 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C. The virion-containing suspension (1 ml) was then treated with 40 U of RQ1 RNase-free DNase (Promega, Madison, Wis.). After 1 h of incubation at 37°C, the DNase was removed by isolating the virions via centrifugation, as described above. Endogenous reverse transcription was then initiated in a simple buffer system:

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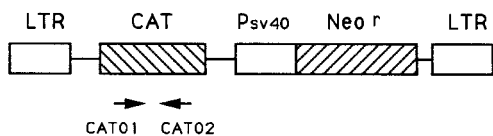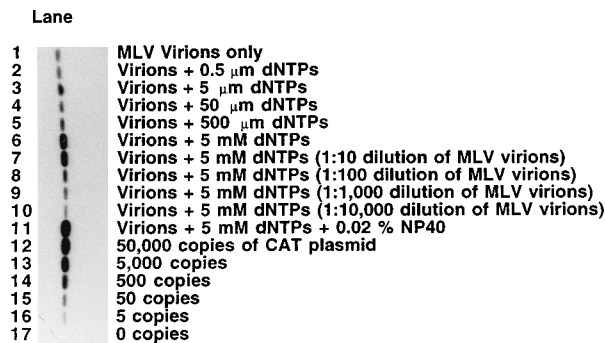
FIG. 1. Schematic map of the murine retroviral vector (pLXSN-CAT) used in these studies and the location of the primer pair for quantitative DNA PCR analyses. The construction of this plasmid has been previously described (11). The 5′ MLV long terminal repeat (LTR) is used to express the CAT gene product, while the neomycin resistance gene product (Neo$^r$) is expressed from a simian virus 40 promoter (Psv 40).



FIG. 2. Endogenous reverse transcription within recombinant murine retroviral virions. DNase-treated virions were mixed in a simple reaction mixture with dNTPs at various concentrations (36). After a 2-h incubation at 37°C, the reaction was stopped by boiling. The viral DNA was extracted and subjected to PCR for 30 cycles, with CAT 01 and CAT 02 as the primer pair. The PCR products were separated on a 1.5% agarose gel, transferred onto a nylon filter, and subsequently detected with a $^{32}$P-labelled probe (CAT 03) via Southern blotting. A serial dilution of known copy numbers of the CAT-expressing plasmid pLXSN-CAT was analyzed under the same conditions (lanes 12 to 17) and was used as a positive control to generate a standard curve. The autoradiograph is representative of at least two independent experiments performed in duplicate.

10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2.4 mM MgCl$_2$, and dNTPs (Pharmacia, Inc., Piscataway, N.J.) at various concentrations. The reaction was allowed to proceed for 2 h at 37°C. After boiling for 10 min to stop the reaction, the viral DNA was extracted and amplified by PCR, as previously described (35, 36).

In brief, the virion-associated DNA was extracted with a lysing buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1% sodium dodecyl sulfate, proteinase K [100 μg/ml]) prior to phenol-chloroform (1:1) extraction and ethanol precipitation. After extraction, 25 μl of each of the samples was added to 25 μl of a PCR mixture (5 mM MgCl$_2$, 220 μM dNTP, 50 pmol [each] positive- and negative-strand primers, 2 U of *Taq* DNA polymerase [Perkin-Elmer Cetus, Foster City, Calif.], 5 μl of *Taq* buffer). The samples were then subjected to 30 cycles of PCR consisting of 2 long denaturation cycles during which the temperature was maintained at 94°C for 3 min, decreased to 53°C over a 3-min period, and finally increased to 72°C and maintained for 2 min. Following the first 2 cycles, the remaining 28 cycles consisted of the same temperatures being maintained for 30 s each. The reactions were performed on an automated DNA thermocycler (Perkin-Elmer Cetus). The primer pair used to amplify the CAT gene was CAT 01 (5′-GCAATGAAAGACGGTGAGCTG-3′, nucleotides 449 to 469) and CAT 02 (5′-AGACGCCACATCTTGCGAATA-3′, nucleotides 581 to 601) (3). The PCR products were analyzed by Southern blot analysis with a $^{32}$P-labelled probe, CAT 03 (5′-ACTGAAACGTTTTCATCGCTCTGGAGTGAATAC-3′, nucleotides 518 to 550) (3).

Figure 2 demonstrates that there was some DNase-resistant viral DNA within the virions, even without the addition of exogenous dNTPs (lane 1), which is consistent with previous findings (20, 31, 35–37). The de novo-synthesized intravirion reverse transcripts were primarily expressed when exogenous dNTPs reached a relatively high concentration (5 mM) (Fig. 2, lane 6). Serial dilution of viral DNA, prepared prior to PCR, indicated that the increment of viral DNA driven by the dNTPs was approximately 100-fold (Fig. 2, lane 8). As expected (6, 33), more viral DNA was synthesized when the nonionic detergent Nonidet P-40 was added at optimal concentrations (Fig. 2, lane 11).

To assess the functional impact of endogenous reverse transcription upon the infectivity of these recombinant MLV virions, the viruses were produced as described above. After elimination of cells and cellular debris by centrifugation at 500 × *g* for 10 min and subsequently at 5,000 × *g* for another 10 min, the virion-enriched supernatants of virus-producing cells were treated with 2.4 mM MgCl$_2$, with or without 5 mM dNTPs, at 37°C for 2 h. The virions were serially diluted by 10-fold increments and added into the wells of 24-well plates containing 4 × 10$^4$ NIH 3T3 cells per well along with 5 μg of Polybrene per ml. After incubation for 16 h at 37°C, the cells were vigorously washed three times with PBS, and fresh Dulbecco's

modified Eagle's medium plus 10% fetal calf serum was added to the cultures. Thereafter, the viral infectivity was measured via three complementary assays. These included (i) quantitative PCR assays for proviral DNA copy numbers within infected cells, (ii) Assays of CFU, and (iii) CAT assays for expression of the transduced gene. Firstly, for quantitative PCR assays to detect proviral DNA in infected cells, cells were replated at 20% confluence every 3 to 4 days postinfection. At 14 days postinfection, the cells were collected, the DNA was extracted, and proviral DNA was amplified by PCR, as previously described (36). Secondly, for the CFU assay, G418 (400 μg/ml, active) (Gibco-BRL, Gaithersburg, Md.) was added to the cultures, at 24 h postinfection. The G418-resistant CFUs were then counted via light microscopy after 14 days. Finally, for CAT analysis, the cells were washed with PBS at 3 days postinfection and then collected with a rubber cell scraper in 300 μl of 250 mM Tris-HCl (pH 7.8). CAT assays were performed as previously described (14).

Quantitative PCR analysis of proviral DNA in the target cells at 14 days postinfection indicated that more proviral DNA was synthesized in the cells when the input viruses were treated with dNTPs than when they were untreated. By either copy number analysis or endpoint estimation, this increase was approximately 100-fold (Fig. 3). Further, the expression of the two reporter genes in the retroviral vector was approximately 10-fold higher when the input virions were treated with dNTPs, as measured by CAT assays (Fig. 4). Likewise, CFU analyses yielded 6.4 × 10$^6$ and 4.5 × 10$^6$ CFU when dNTPs (5 mM) were added and 6.8 × 10$^5$ and 5.0 × 10$^5$ CFU without dNTPs (results of two independent experiments). The percentages of the conversions of chloramphenicol in the CAT assays were approximately 20 and 2% for undiluted virions, either treated with dNTPs or untreated, respectively (Fig. 4, lanes 1 and 2).

As illustrated in Fig. 1, the CAT gene product was expressed by the 5′ MLV long terminal repeat, while the neomycin resistance (Neo$^r$) gene was driven by a simian virus 40 promoter. Thus, these genes should be expressed independently of one another. The increased expression of both genes, at the same level, suggests that the copy number of their proviral DNA was
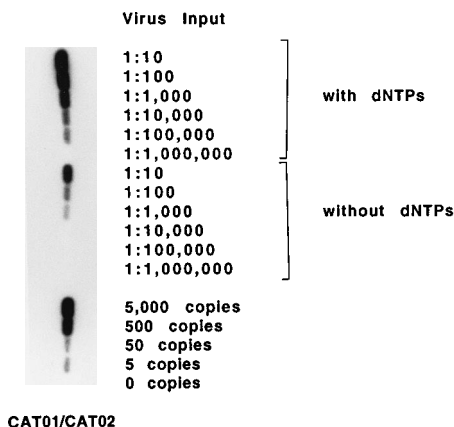
FIG. 3. Proviral DNA in cells infected with the murine retroviral vector. Virions treated with 5 mM dNTP and untreated virions were serially diluted and mixed with target NIH 3T3 cells. At 14 days postinfection, the cells were collected and the DNA was extracted and then subjected to PCR for 30 cycles, with CAT 01 and CAT 02 as primers. The PCR products were detected with the $^{32}$P-labelled CAT 03 probe and Southern blotting. The standard curve of the serially diluted CAT-expressing plasmid is presented. The autoradiograph is representative of two independent experiments performed in duplicate.

increased. It should be noted that the increment for the expression of two reporter genes is less than that for the proviral DNA synthesis in the target cells. The difference may be due to some viral DNA synthesized within the virions which is not suitable for integration into the host cell's chromosomal DNA. Without integration, the genes carried by murine retroviral vectors may not be effectively expressed (28). Clearly, only relatively few copies of retroviral reverse transcripts lead to transcriptionally active proviral species.

In addition to data from previous studies (4, 6, 10, 30, 33, 36), our data further demonstrate that endogenous reverse transcription, without nonphysiological virion permeabilization, may be a relatively common phenomenon for different groups of retroviruses. Even though reverse transcription is more efficient in the presence of detergents, significant quantities of intravirion DNA can be synthesized without detergent and can subsequently augment viral infectivity. It has been suggested that the cellular membrane may not be significantly permeable to either ribonucleoside triphosphates or dNTPs (19, 25, 29, 32). The retroviral envelope is derived from the cellular membrane of virus-producing cells. Therefore, it might be assumed that the viral envelope is not permeable to dNTPs.

However, recent studies regarding the composition of retroviral envelopes indicated that there are some differences between cellular membranes and viral envelopes (1, 2). These differences may lead to the permeabilization of retroviral envelopes to dNTPs. There may also be some minor injuries to the viral membranous envelope, such that dNTPs can enter the virions without detergent. Nevertheless, the data presented here indicate that endogenous reverse transcription for recombinant MLV occurs only when the dNTP concentration is relatively high. A similar phenomenon has been demonstrated in another type C retrovirus, human T-cell leukemia (or lymphotropic) virus type I (unpublished data). In contrast, our previous data demonstrated that the endogenous reverse transcription in human immunodeficiency virus type 1 virions could occur when dNTP concentrations are relatively low (36 and unpublished data). The requirement for various concentrations of dNTPs to drive endogenous reverse transcription by different retroviruses may be due to differences in the various reverse transcriptase enzymes or the structure of the virions.

As mentioned above, the products of retroviral endogenous reverse transcription may directly integrate into chromosomal DNA and/or take a shorter time to complete reverse transcription, compared with virions which harbor only genomic RNA (35, 36). Further, a virion-derived structure, the nucleoprotein complex, may be necessary for viral DNA synthesis, especially for efficient template switching during reverse transcription (7–9). If this replicative machinery remains in a target cell for a significant time, it may begin to degrade prior to completion of reverse transcription. Thus, the efficiency of reverse transcription will be affected. Therefore, those viruses that enter non-S-phase cells harboring relatively low concentrations of dNTPs in the cytoplasm (17, 18, 26) might gain significant benefit from the reverse transcription before entering the target cells. It is unlikely that the increased infectivity of these retroviruses is due to the increase of intracellular dNTP concentration by residual dNTPs in the supernatants because (i) the concentration of dNTPs after serial dilution is even lower than that found in quiescent cells (12) and (ii) dNTPs may not efficiently enter target cells, as discussed above. In addition, although it remains formally possible that dNTPs increase retroviral particle stability, this appears unlikely for a 2-h incubation period at 37°C.

The studies described in this report illustrate a quick, simple, and efficient method for increasing the transduction efficiency of certain retroviral vectors. As such, it may prove useful for specific gene therapeutic techniques. It may also be worthwhile to further investigate the effects of alterations of infectivity of retroviruses upon initially quiescent cells, after initiating endogenous reverse transcription. It is possible that the virions initiating endogenous reverse transcription will have kinetic advantages during infection of initially quiescent cells, prior to stimulation. Further, to obtain higher titers of infectious murine retrovirus, it may be valuable to search for alternative methods to increase the efficiency of endogenous reverse transcription and infectivity, in addition to the use of dNTPs (16). Moreover, as mentioned above, the molecular structure of de novo-synthesized intravirion viral DNA should be further evaluated to fully understand the molecular mechanism(s) involved in altering retroviral infectivity.
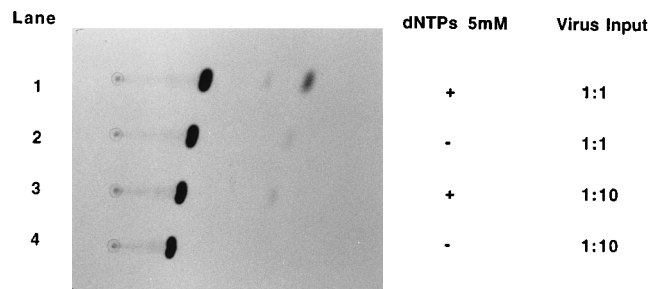


FIG. 4. CAT activity in cells infected with the retroviral vector. The viruses treated with 5 mM dNTPs and untreated viruses were serially diluted and used to infect the target NIH 3T3 cells. At 3 days postinfection, the cells were collected for CAT analyses, as previously described (14). Quantities of total protein in all reaction mixtures were standardized. Data are representative of two independent experiments performed in duplicate.

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