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# Modification of Human Immunodeficiency Virus Type 1 Reverse Transcriptase to Target Cells with Elevated Cellular dNTP Concentrations*

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**Retroviruses and DNA viruses utilize cellular dNTPs as substrates for their DNA polymerases during viral replication in infected cells. However, because of S phase-dependent dNTP biosynthesis, the availability of cellular dNTPs significantly varies among cell types (*e.g.* dividing *versus* nondividing cells and normal *versus* tumor cells). Here we tested whether alterations in the dNTP utilization efficiency and dNTP binding affinity of viral DNA polymerases can switch viral infection specificity to cell types with different dNTP concentrations. We employed an HIV-1 reverse transcriptase (RT) mutant (Q151N), which is catalytically active only at high dNTP concentrations because of its reduced dNTP binding affinity. Indeed, the modified HIV-1 vector harboring the Q151N mutant RT preferentially transduced tumor cells containing higher cellular dNTP concentrations than primary cells (*e.g.* human lung fibroblasts (HLFs) and human keratinocytes). Although the wild type HIV-1 vector transduced both HLFs and tumor cells, the Q151N vector failed to transduce HLFs and keratinocytes but efficiently transduced tumor cells. Pretreatment of HLFs with deoxynucleosides, which increase cellular dNTP pools, enabled the mutant vector to transduce HLFs, suggesting that the transduction failure of the RT mutant vector to primary cells is because of inefficient reverse transcription in low cellular dNTP environments. We also observed that the Q151N vector expressing herpes simplex virus-thymidine kinase renders tumor cells sensitive to gancyclovir. This study validates a novel strategy in which modifications of viral DNA polymerases in various vector systems allow the delivery of target genes exclusively to tumor cells exploiting elevated cellular dNTP concentration as a tumor cell-specific host factor.**

Lentiviruses, including human immunodeficiency virus type 1 (HIV-1),[3] are unique in their ability to infect terminally differentiated or nondividing cells such as macrophage and microglia, as well as dividing cells such as activated CD4+ T cells (1, 2). Other retroviruses such as oncoretroviruses productively infect only dividing cells (1, 3). Because of their ability to infect nondividing cells, lentivirus-based vector systems have been developed as useful gene delivery tools for many nondividing cell types such as neuronal cells (4–6). Several DNA viruses such as adenovirus and herpes simplex virus (HSV) also have the capability to replicate in nondividing cells and have been developed as vector systems targeting nondividing cells (7–9).

Cellular deoxynucleoside triphosphates (dNTPs) serve as substrates for enzymatic DNA synthesis of retroviruses, as well as many DNA viruses, and virally encoded DNA polymerases (*e.g.* reverse transcriptase for retroviruses) catalyze this replication process. Numerous studies have demonstrated that cancer cells have a higher dNTP content compared with normal dividing cells because of their uncontrolled cell division (10–14). Because the dNTP concentration varies between cell types, the proviral DNA synthesis rate of retroviruses could depend on the cell type that the virus infects. Using a highly sensitive dNTP assay that we recently established (2), we determined the dNTP concentrations of human macrophage and CD4+ T cells, two target cell types of HIV-1. We found that human macrophage (~30 nM) have ~150–225 times lower dNTP concentrations than activated CD4+ T cells (~5 $\mu$M). Despite the wide range of dNTP concentrations found in HIV-1 target cells, we found that HIV-1 RT efficiently synthesizes DNA even at concentrations as low as 50 nM because of its tight binding affinity for dNTP substrates (15).

We recently isolated two HIV-1 RT mutants, V148I and Q151N, that specifically exhibit reduced dNTP binding affinity ($1/K_d$) without a change in catalysis ($k_{pol}$) (16, 17). Kinetic and structural analyses suggested that these mutations interfered with H-bond formation between the side chain of the Gln-151 residue and the 3′-OH group of the incoming dNTP substrate (18). These two mutant RTs showed greatly reduced DNA synthesis at the low dNTP concentrations found in macrophage but are as active as wild type at high dNTP concentrations (2). Furthermore, HIV-1 vectors containing these dNTP binding mutant RTs failed to transduce macrophage but efficiently infected HeLa cells, which contain high dNTP levels (2). Therefore, unlike the wild type HIV-1 vector, whose infectivity is little affected by cellular dNTP concentration, the transduction of these RT mutant vectors appears to depend on the availability of cellular dNTP substrates in the cells that they infect.

Specific gene delivery by replication-incompetent viral vectors to cancer cells includes the delivery and expression of the following: 1) toxic proteins (19); 2) correct forms of missing or altered gene products responsible for tumorigenesis (*e.g.* p53) (20); and 3) RNA interference blocking the expression of essential genes (21, 22). Viral vector-based cancer gene therapy includes the use of well characterized animal virus systems such as adenoviruses, adeno-associated viruses, retroviruses, and herpesviruses (4, 6). Another anti-cancer gene therapy strategy is the use of oncolytic viral vectors that conditionally replicate only in tumor cells containing tumor-specific genetic alterations. Examples are

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[3] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; RT, reverse transcriptase; HLF, human lung fibroblasts; dNs, deoxynucleosides; HSV-TK, herpes simplex virus-thymidine kinase; GCV, gancyclovir; LTR, long terminal repeat; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; EGFP, enhanced GFP; m.o.i., multiplicity of infection; WT, wild type.

the E1B-deleted adenovirus that replicates only in cells with a loss of p53 function and induces cell killing (23) and adenovirus expressing E1A from the telomerase reverse transcriptase promoter that is usually activated in tumor cells (24). These strategies focus on minimizing vector transduction to normal primary cells. However, some of these tumor-specific factors are not always found in all tumor cells (*e.g.* loss of p53 function) (23), which restricts the utility of these oncolytic vectors. Therefore, identification of new and universal tumor-specific alterations that are essential to transduction and replication of the viral oncolytic vector systems will improve the efficacy and safety of anticancer vector systems.

In this study, we tested whether the modification of viral DNA polymerases, which makes them active only at high dNTP concentrations, can restrict the infectivity of the viral vector systems specifically to cell types containing high dNTP concentrations. For this test, we employed an HIV-1-based vector system harboring a mutant HIV-1 RT with reduced dNTP binding affinity as a model system. Indeed, this modified HIV-1 vector was able to deliver genes specifically to cells containing high dNTP concentrations such as cancer cells with minimum infection to normal primary cells containing relatively low cellular dNTP concentrations such as human lung fibroblasts, keratinocytes (this study), and macrophage (2).

## MATERIALS AND METHODS

*Plasmids, Cells, and Chemicals—Escherichia coli* DH5α (Stratagene) was used for the construction and amplification of plasmids, and BL21(DE3) (Novagen) was used for the overexpression of HIV-1 RT proteins. The HIV-1 packaging-GFP plasmid, pHIV-GFPΔEnv (a gift from Dr. Vicente Planelles, University of Utah), expresses all strain NL4-3 proteins except for Env and Nef, which have been replaced by EGFP (25). A plasmid expressing vesicular stomatitis virus envelope protein, pVSV-G, was also obtained from Dr. Planelles. The plasmid encoding herpes simplex virus thymidine kinase (HSV-TK) was obtained from Dr. L. A. Loeb (University of Washington). The CHME5 cell line, a human fetal microglia cell line transformed by SV40 large T antigen (26), was used for viral titering and was a gift from Dr. David Mock (University of Rochester). CHME5 cells were cultured in Dulbecco's modified Eagle's medium from CellGro containing 5% fetal bovine serum (HyClone). The A549 human lung epithelial tumor cell line was a gift from Dr. Toru Takimoto (University of Rochester), and these cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The 293 FT cell line, obtained from Invitrogen, and the PANC1 cell line, obtained from ATCC, were both grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The primary human lung fibroblasts were received as a gift from Dr. Richard Phipps (University of Rochester) and were cultured in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) as described previously (27). Restriction enzymes, T7 RNA polymerase, and T4 polynucleotide kinase were purchased from New England Biolabs.

*Purification of HIV-1 RT Proteins*—The wild type and Q151N HIV-1 RT proteins were overexpressed in *E. coli* BL21 (Novagen, WI) as described previously (28). The HIV-1 RT expression construct encodes the full-length HIV-1 RT (HXB2) with a hexahistidine tag at the N terminus. The hexahistidine-tagged RT was purified using $Ni^{2+}$ chelation chromatography as described previously (17, 29, 30). From 1 liter of culture, we were able to purify 4 mg of p66/p66 homodimers. To examine the purity of the RT proteins, 4 μg of the purified RTs were analyzed by 10% SDS-polyacrylamide gels using 4 μg of 98% pure bovine serum albumin (Sigma) as a control. The gels visualized by Coomassie staining were analyzed by a densitometer, and the purified RT proteins showed similar levels of minor contaminants as the bovine serum albumin control, suggesting that the RT proteins used in this study must be at least 95% pure.

*Assays for dNTP Concentration-dependent DNA Polymerase Activity of HIV-1 RT Proteins*—A 182-nucleotide-long RNA template encoding the HIV-1 *pol* sequence was generated by *in vitro* run-off transcription from the BamHI-linearized pol donor construct pNL-RT3612-3773 (31) using T7 RNA polymerase as described previously (31). The full-length transcript was purified on a 6% denaturing polyacrylamide gel. The 19-nucleotide DNA primer MB22 (5′-GCTTGCCAATACTCT-GTCC-3′), complementary to the 3′ end of the pol RNA template, was 5′-end-labeled using T4 polynucleotide kinase and [γ-$^{32}$P]ATP (Amersham Biosciences). Labeled primer (50 fmol) was then mixed with RNA template (100 fmol) in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM KCl and was incubated at 95 °C for 1 min. The template/primer mixture was slow-cooled to room temperature to facilitate primer annealing and refolding of the RNA template. HIV-1 RTs with similar levels of activity (RTs giving 50% primer extension at high dNTP concentration) were preincubated for 2 min at room temperature with primer-template, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol in a total 12-μl reaction volume. Reactions were initiated by the addition of 6 mM $MgCl_2$ and deoxynucleoside triphosphates at varying concentrations (5–0.05 μM). After incubation at 37 °C for 5min, reactions were terminated by the addition of 12 μl of termination buffer (90% formamide, 10 mM EDTA, pH 8.0, and 0.1% xylene cyanole and 0.1% bromphenol blue). Reaction products were resolved on 6% polyacrylamide-urea gels and visualized by a PhosphorImager using ImageQuant software (Amersham Biosciences).

*HIV-1 RT-based dNTP Assay with Cellular dNTP Samples*—The protocols for dNTP extraction from cells and the dNTP assay were performed as described previously (2). dNTPs extracted from tumor cells and primary cells were resuspended to 100 and 10 μl per $1 \times 10^6$ cells.

*Pseudotyped HIV-1 Vector Production*—pHCMV-VSVG envelope vector (32) and pHIV-EGFPΔEnv transfer vector (25), which encodes the HIV-1 NL4-3 genome with the EGFP gene in place of HIV-1 Nef and a deletion of the *env* gene, were used for preparing pseudotyped HIV-1. The Q151N mutations were cloned into the pD3-EGFP (pNL4-3-based) vector using overlapping PCR with proper primers that were designed based on the pNL4-3 RT sequence as described previously (2). Production of pseudotyped HIV-1 vectors was then performed as described previously (2). p24 levels of produced vectors were measured using the HIV-1 p24 antigen enzyme-linked immunosorbent assay test system (Beckman Instruments).

*Vector Infections and Analysis*—All cell types were treated with 10 μg/ml Polybrene prior to transduction with either WT or Q151N virus at an m.o.i. of 5. 48 h post-transduction, cells were washed and visualized by fluorescence microscopy (Leica), using EGFP as an indicator of transduction efficiency. Following imaging, cells were trypsinized and fixed in a 0.5% formaldehyde solution for further analysis by FACS. Cell samples were analyzed by FACS using the FL-1 channel to detect EGFP expression, and the percentage of transduction was determined using the Cellquest program (version 3.3; BD Biosciences).

*dN Effect Assay*—Prior to transduction, cells were treated with 2.5 mM each dN (dA, dC, dT, and dG; Sigma). After 4 h of incubation, Polybrene was added at 10 μg/ml, and cells were transduced with either wild type or Q151N vector at an m.o.i. of 5. 24 h post-transduction, cells were washed with Dulbecco's phosphate-buffered saline, and fresh medium was added. Cells were then incubated for an additional 24 h

before imaging and FACS analysis. All assays were performed in triplicate.

*2LTR Circle PCR*—Primers for the amplification of the HIV-1 2LTR circle DNA were described previously (33, 34). The forward primer anneals to the 75 bp upstream from the 3′ end of the 5′-LTR region of NL4-3 (5′-GTGCCCGTCTGTTGTGTGACT-3′), and the reverse primer anneals to the 33 bp downstream of the 5′ end of the 3′-LTR region (5′-CTTGTCTTCTTTGGGAGTGAATTAGC-3′), and the probe (5′-6-carboxyfluorescein-TCCACACTGACTAAAAGGGTCT-GAGGGATCTCT-carboxytetramethylrhodamine-3′) anneals at the junction of 5′–3′-LTR region. The human lung fibroblast cells with or without dN (2.5 mM) treatment for 4 h were incubated with wild type or Q151N vectors with m.o.i. of 5. The cells were harvested at 6, 12, and 24 h post-transduction. The total cellular DNA was extracted using a genomic extraction kit (Qiagen). The amplification was performed with the forward primer (0.9 $\mu$M), the reverse primer (0.9 $\mu$M), the probe (1 $\mu$M), and 300 ng of the extracted cellular DNA using 2× Taqman universal mix (Applied Biosystems). PCRs were initially incubated at 50 °C for 2 min followed by 95 °C for 15 min. Each cycle of the PCR (50 cycles) was then performed at 95 °C for 15 s followed by 60 °C for 1 min. Standard curves for the quantification of 2LTR were generated by serial dilution of a known concentration of 2LTR DNA. Standard curve was linear within $10^1$ to $10^6$ copies of 2LTR DNA.

*Coculturing of Primary and Tumor Cells*—To culture different pairs of cell types, the medium of the primary cell type, not the tumor cell line, was used. For coculturing of the human lung fibroblasts and the lung epithelial tumor cell line A549s, cells were cultured in minimal essential medium (Invitrogen) containing 10% fetal bovine serum and transduced with the appropriate vector as described above.

*HSV-TK Vector Construction and Gancyclovir (GCV) Sensitivity Measurement*—Wild type and Q151N mutant RT vectors expressing HSV-were constructed by replacing the EGFP gene with the HSV-TK gene, yielding pHIV-HSVTKΔEnv. After production of WT-HSV-TK and QN-HSV-TK vector as described above, the p24 levels were measured by the HIV-1 p24 enzyme-linked immunosorbent assay. Cells were transduced as described above with either WT-HSV-TK or QN-HSV-TK virus at identical p24 activities. After 48 h post-transduction, cells were washed with Dulbecco's phosphate-buffered saline and either left untreated or treated with two concentrations of GCV, 5 and 40 $\mu$g/ml. Cells were treated with GCV for a period of 5 days, changing the media and adding fresh GCV every 24 h. After 5 days, cells were trypsinized and counted using a hemocytometer. The survival of cells following GCV treatment was normalized as a percentage of the cell number of untreated cells transduced with the same viral vectors.

## RESULTS

*dNTP Concentration-dependent DNA Polymerization of HIV-1 RT Proteins*—We recently isolated an HIV-1 RT mutant, Q151N, which exhibits reduced binding affinity to dNTP substrates without altering catalysis (17). Because of its reduced dNTP binding affinity, the dNTP binding step of this mutant, which is fast at high dNTP concentrations ($\mu$M range), becomes rate-limiting at low dNTP concentrations (*e.g.* 50 nM). We examined the dNTP concentration-dependent DNA polymerase activity of wild type and Q151N HIV-1 RTs using a primer extension assay. This assay employed a 5′-$^{32}$P-labeled 19-mer primer (Fig. 1, *S*) annealed to a 182-nucleotide-long RNA template encoding the *pol* gene of HIV-1 and varying concentrations of dNTPs, which can yield fully extended products (Fig. 1, *F*). First, the amount of RT protein showing ~50% primer extension at 5 $\mu$M was determined (Fig. 1, *5*). The concentrations of the wild type and Q151N RT proteins showing 50% primer extension at high dNTP concentrations were very similar (50–60 nM). Next, the same reactions were repeated with decreasing concentrations of dNTPs. As shown in Fig. 1, the Q151N mutant showed a dramatic decrease in primer extension even at relatively high

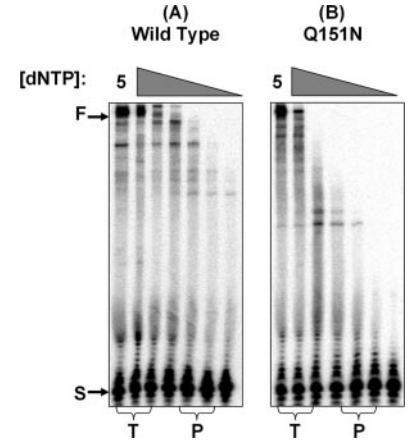
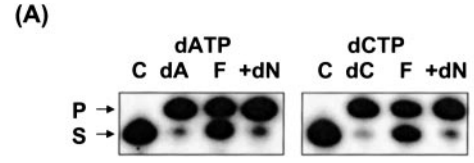


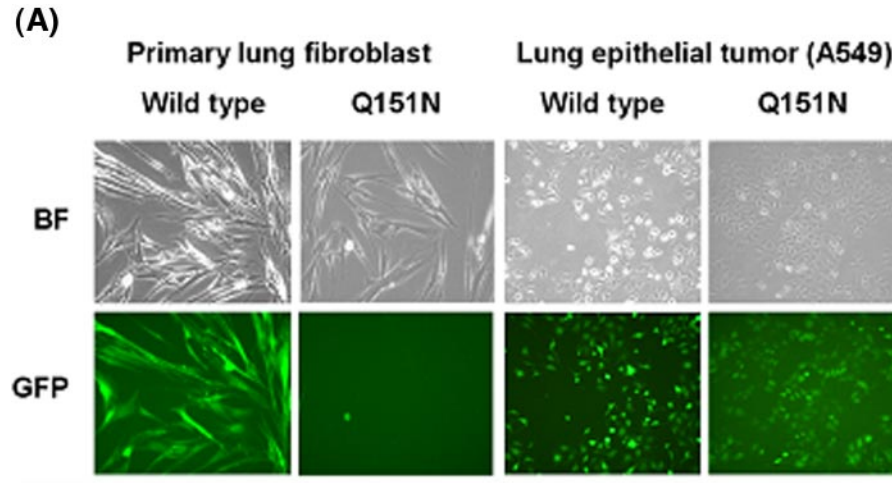

FIGURE 1. **dNTP concentration-dependent reverse transcription activity of HIV-1 RT proteins.** A $^{32}$P-labeled 19-mer primer (*S*) annealed to a 182-nucleotide-long RNA template encoding a part of the HIV-1 *pol* gene was extended by wild type (*A*) and Q151N mutant (*B*) HIV-1 RT proteins (50–60 nM) showing 50% of the full extension in a 5-min reaction (*F*) at 5 $\mu$M dNTPs (*1st lane*). Similar reactions were repeated with decreasing concentrations of dNTP: *1st to 7th lanes*, 5, 2.5, 1, 0.5, 0.25, 0.1, and 0.05 $\mu$M, respectively. The reactions were analyzed by 6% denaturing gels. The dNTP concentrations observed in tumor/transformed cells (*T*) and primary cells (*P*) are marked (see Fig. 2).

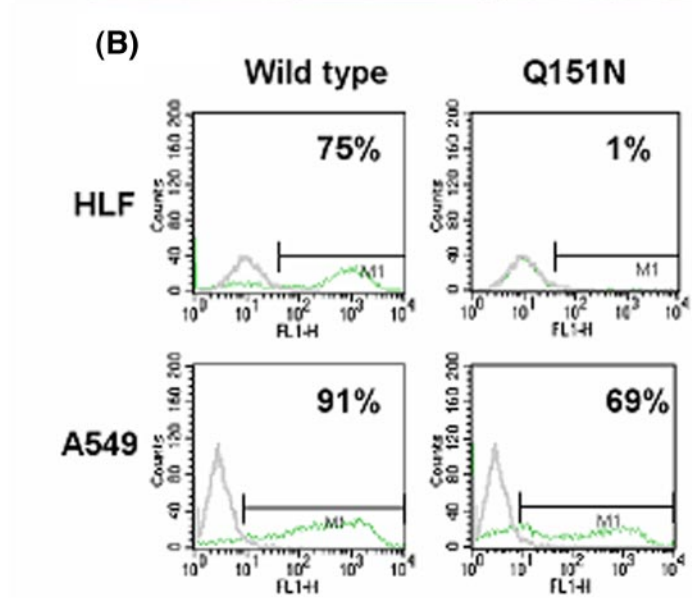



(B)

| Human cell types | [dNTP] μM |
|---|---|
| Primary lung fibroblast | 0.1~0.2 |
| Primary keratinocyte | 0.1~0.6 |
| A549 | 1.7~3.3 |
| PANC1 | 1.3~2.4 |
| CHME5 | 2.3~5.7 |
| 293FT | 1~2.3 |
| Lung fibroblast with dNs | 20~90 |

FIGURE 2. **dNTP concentrations of human cells.** *A,* HIV-1 RT-based dNTP assay: A $^{32}$P-labeled 18-mer primer (*S*) annealed to a 19-mer template was extended by HIV-1 RT with various dNTP sources, producing the 19-mer product (*P*). *C lane,* no dNTP; *2nd lane*, 250 $\mu$M pure dATP or dCTP; *3rd lane*, dNTP extracted from primary HLFs ($4 \times 10^4$ cells); *4th lane*, HLF with 2.5 mM dN treatment ($4 \times 10^4$ cells). *B,* dNTP concentrations of human cell types; amounts of dNTPs contained in each cell type were calculated by plotting the % of the primer extension obtained from the dNTP assay to the standard curves derived from the dNTP assay reactions with known amounts of pure dNTPs (2). The dNTP samples from the dN-treated HLFs were properly diluted to obtain primer extension in the linear range (4–128 fmol) (2). The volume of each cell type used in this study was measured by confocal microscopy for determining the cellular dNTP concentration of each cell type. The assays were performed in triplicate, and S.D. of each dNTP was <25%. The ranges of the dNTP concentrations indicate the variations between the four dNTPs.




(C)

| Cell Type | WT vector % transduction | Q151N vector % transduction (Ratio to wild type vector) |
|---|---|---|
| CHME-5 | 83 | 83 (1.00) |
| A549 | 91 | 69 (0.76) |
| PANC-1 | 62 | 65 (1.05) |
| 293 FT | 46 | 31 (0.67) |
| Human lung fibroblast | 75 | 1 (0.01) |
| Human keratinocyte | 76 | 2 (0.02) |

FIGURE 3. **Transduction of HIV-1 vectors containing wild type and Q151N RTs to normal and tumor cells.** *A,* primary HLFs and A549 human lung epithelial tumor cells were transduced by an equal p24 amount ($1.5 \times 10^6$ pg) of wild type and Q151N HIV-1 vectors. The EGFP expression of transduced cells was analyzed at 48 h post-transduction by fluorescence microscopy. Both bright field (*BF*) and dark field (*GFP*) images are shown. *B,* HLFs and transformed human cell lines were transduced with equal p24 amounts of wild type and Q151N vectors ($3 \times 10^5$ pg for CHME5), and the transduction efficiency for each cell type was determined by FACS. The untransduced cells are shown in *gray*, and vector-transduced cells are depicted by the *green* histograms. Parameters for gating were set so that the untransduced controls gave only 1% GFP-positive cells according to the FL-1 channel (*x* axis). Total cells were gated on the basis of physical parameters (forward and side scatter), and the percent GFP-positive cells was determined. The *y* axis corresponds to the number of cells counted during FACS analysis. *C,* the average transduction efficiency of each experiment performed in duplicate and the transduction efficiency ratios between wild type and Q151N mutant vectors. Standard deviation was <10% in multiple measurements.

dNTP concentrations (*e.g.* Fig. 1, *3rd lane,* 1 μM), whereas the wild type HIV-1 RT maintained significant levels of primer extension even at concentrations as low as 50–250 nM. This experiment demonstrates that the Q151N HIV-1 RT dNTP binding mutant is capable of performing efficient reverse transcription only at high dNTP concentrations such as 2.5 and 5 μM (Fig. 1, *1st* and *2nd lanes*).

*Cellular dNTP Concentrations of Normal Primary Cells and Tumor Cells*—Employing a highly sensitive dNTP assay that we established recently (2), we determined the cellular dNTP concentrations of normal primary HLFs, primary human keratinocytes, and four human tumor cell lines (a lung epithelial tumor cell line (A549), an SV40-transformed human fetal microglia cell line (CHME5), an

SV40-transformed human kidney epithelial cell line (293FT), and a human pancreatic epithelial cancer cell line (PANC1)). The dNTP assay is a single nucleotide incorporation reaction using a $^{32}$P-labeled 18-mer primer (Fig. 2*A, S, 1st lane*, no dNTP) annealed to a 19-mer DNA template. These template/primers contain single nucleotide 5′ template overhangs (see "Materials and Methods"). The four different templates contain a different sequence in their 5′ overhang, which can be enzymatically filled with one of the four natural dNTPs from mixtures extracted from the cell types discussed above, producing a 19-mer product (Fig. 2*A*, *P, 2nd lane,* with 2.5 mM pure dNTPs). This assay uses HIV-1 RT as a DNA polymerase and cellular dNTPs extracted from $\sim 4 \times 10^4$ cells (Fig. 2*A, 3rd lane*). Using standard curves obtained with known amounts of each dNTP and cell volumes determined using confocal microscopy (2), the cellular dNTP concentrations of HLFs and the four human cancer/transformed cell lines were determined. As shown in Fig. 2*B*, the human tumor/transformed cell lines tested in this study have ~10–30 times higher dNTP concentrations (1–5.7 $\mu$M) than HLFs (0.1–0.2 $\mu$M) or primary human foreskin keratinocytes (0.1–0.6 $\mu$M). Note that the Q151N HIV-1 RT mutant protein displayed a dramatically decreased DNA polymerase activity at the dNTP concentrations equivalent to those observed in HLFs and keratinocytes, whereas wild type HIV-1 RT still remained relatively active at these low dNTP concentrations (Fig. 1).

*Transduction Efficiency of HIV-1 Vectors Containing Wild Type and the Q151N dNTP Binding Mutant RT to Various Cell Types*—Next, we tested whether an HIV-1 vector containing the Q151N RT mutant was able to transduce two normal primary human cell types, HLFs and keratinocytes, and four human tumor cell lines containing low and high cellular dNTP concentrations, respectively. The HIV-1 vector system used expresses EGFP, and therefore the transduced cells can easily be detected via fluorescence analysis. First, HLFs and lung epithelial tumor cells (A549s) were individually transduced with the same p24 level of wild type and mutant HIV-1 vectors, and the transduction efficiency was determined at 48 h post-transduction. As shown in Fig. 3, *A* and *B*, the wild type HIV-1 vector successfully transduced both normal primary fibroblasts and epithelial tumor cells with similar efficiency. However, HIV-1 vectors containing the Q151N mutant RT showed very restricted transduction of normal lung fibroblasts, although they efficiently transduced A549s containing high dNTP concentrations (Fig. 3, *A* and *B*). The Q151N vector also showed significantly low transduction efficiency in human keratinocytes, whereas the wild type vector efficiently transduced this primary cell type (Fig. 3*C*). We also observed similar transduction differences between wild type and Q151N vectors in the human pancreatic cancer cell line (PANC1) and the two SV40 T antigen transformed cell lines (CHME5 and 293FT cells) (Fig. 3*C*). Therefore, the data shown in Fig. 3 suggest that, unlike the wild type HIV-1 vector, the mutant vector containing a dNTP binding mutant RT preferentially transduced only cells containing high cellular dNTP concentrations.

*Deoxynucleoside (dN) Effect on the Transduction of HIV-1 Vectors to Primary Cells*—We tested whether the restricted transduction of the Q151N HIV-1 vector to primary human lung fibroblasts was because of the limited dNTP availability in this cell type. For this test, we treated cells with dNs, the precursors of dNTPs, which can enhance cellular dNTP concentrations. First, we determined the dNTP concentration change upon dN treatment of human lung fibroblasts. As shown in Fig. 2*A*, the dN (2.5 mM)-treated human fibroblasts showed a large increase in dNTP levels compared with the untreated cells. The dNTP assay with diluted dNTP samples showed that dN treatment of HLFs increases the dNTP level by approximately several hundredfold. The calculated dNTP concentration of HLFs treated with dNs was 20–90 $\mu$M, which is ~15 times higher than that of the dNTP concentration found in transformed cells (*e.g.* Fig. 2*B, CHME5*). Next, we transduced both dN-treated and untreated human lung fibroblasts with either the wild type or Q151N vector. As shown in Fig. 4, *A* and *B*, the wild type vector transduced both dN-treated and untreated fibroblasts with similar efficiency. As predicted, the Q151N (Fig. 4, *A* and *B*) vector, which showed very restricted transduction in primary lung fibroblasts, showed greatly enhanced transduction upon dN treatment. Next, we examined the proviral DNA synthesis of the Q151N and wild type HIV-1 vectors in HLFs with or without dN treatment using the HIV-1 2LTR circle-specific real time PCR assay (Fig. 4*C*). The 2LTR circle proviral DNA, which is found in nuclei, is an indicator for the completion of HIV-1 proviral DNA synthesis. As seen in Fig. 4*C*, very limited numbers of copies of 2LTR circle DNA were found in untreated HLF cells transduced by the Q151N vector even at 24 h post-transduction, whereas the dN-treated HLFs displayed ~200-fold higher copy numbers. However, the wild type vector showed similar copy numbers of 2LTR circle DNA irrespective of dN treatment, which is consistent with the transduction efficiency demonstrated in Fig. 4*A*. Thus, the data support that the failure of the Q151N HIV-1 vector in the transduction of normal human primary cells is because of the restricted cellular dNTP concentrations in this cell type.

*Transduction of HIV-1 Mutant Vectors to Cocultured Lung Fibroblasts and Epithelial Tumor Cells*—Next, we tested whether the Q151N mutant vectors can preferentially transduce tumor cells containing high dNTP concentrations when tumor cells are cocultured with primary cells containing low dNTP concentrations. For this test, we employed cocultured human lung fibroblasts and human lung epithelial tumor cells, which clearly display their morphological differences. As shown in Fig. 5*A*, the wild type vector efficiently transduced both normal lung fibroblasts (see *arrows*) and lung epithelial tumor cells. However, the same m.o.i. of the Q151N (Fig. 5*B*) vector showed preferential transduction to epithelial tumor cells.

*Specific Killing of Tumor Cells by GCV and Q151N HIV-1 Vector Expressing HSV-TK*—Finally, we tested whether the Q151N HIV-1 vector expressing HSV-TK can induce tumor cell-specific transduction and killing upon treatment with GCV. Gancyclovir is a nucleoside analog that can be phosphorylated by the HSV-thymidine kinase. Phosphorylated GCV is incorporated into the growing nucleotide chain by host DNA polymerase, resulting in chain termination and eventually cell death. For this test, the wild type and Q151N vectors expressing HSV-TK were constructed, and these vectors were used for the transduction of normal human lung fibroblasts and lung epithelial tumor cells with a p24 level equivalent to that of the EGFP-expressing vector used in Fig. 3. Following transduction, cells were either left untreated or treated with GCV at two concentrations (5 or 40 $\mu$g/ml) for 5 days. As shown in Fig. 6*A*, the wild type vector killed both normal human lung fibroblasts and epithelial tumor cells upon GCV treatment. In contrast, when the Q151N vector expressing HSV-TK (Fig. 6*B*) was used for transduction, the majority of the normal human lung fibroblasts survived, whereas the epithelial tumor cells were efficiently killed upon GCV treatment. The data support the tumor-specific killing of the Q151N HIV-1 vector expressing the HSV-TK prodrug.

## DISCUSSION

To improve the tumor cell-specific gene delivery of lentiviral vectors, we employed both a unique tumor-specific host factor, highly elevated

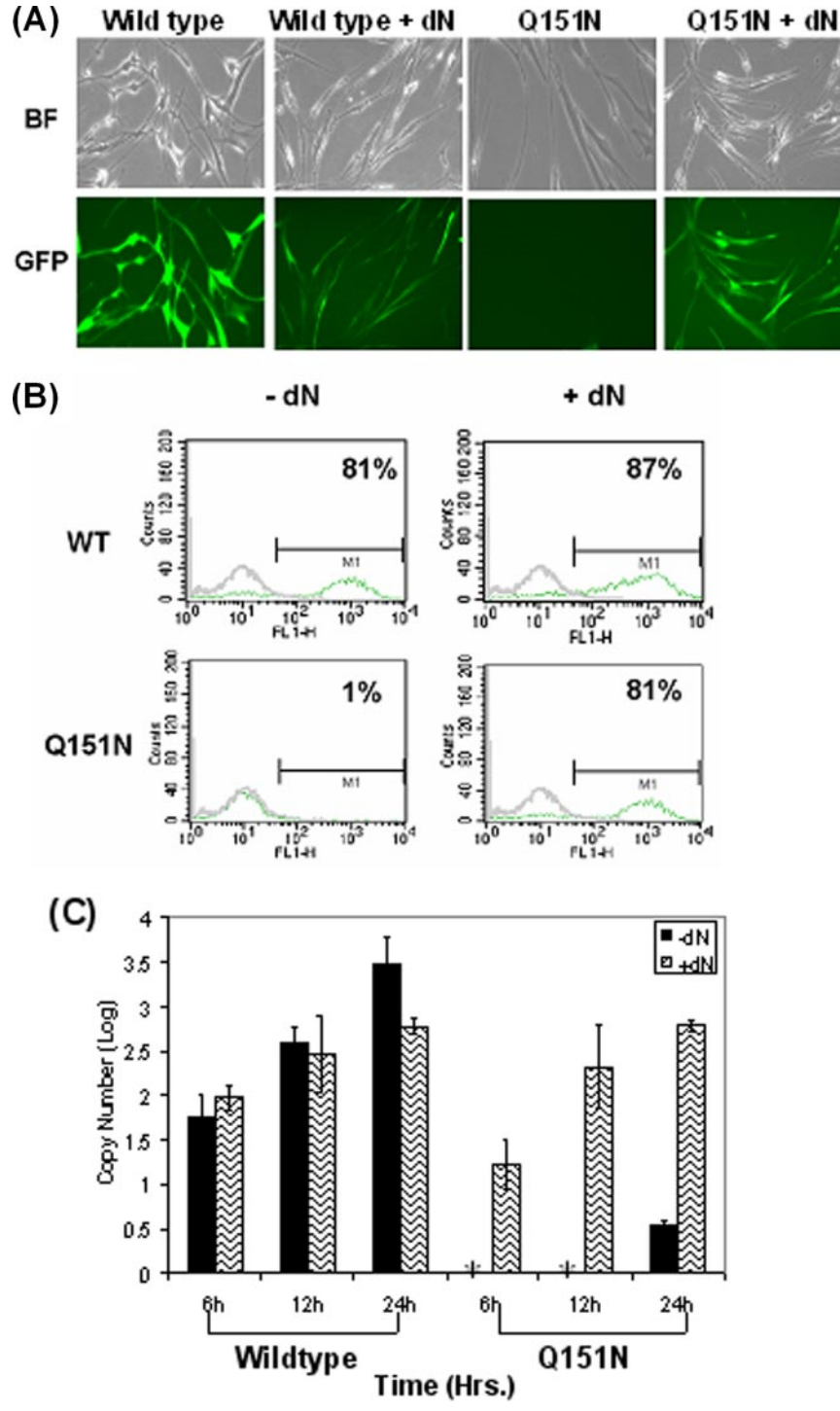



FIGURE 4. **Effect of dN treatment on the transduction efficiency of the wild type and Q151N HIV-1 vectors.** Primary HLFs left untreated or pretreated with 2.5 mM dNs were transduced by an equal p24 level of wild type and Q151N HIV-1 vectors ($1.5 \times 10^6$ pg), and the EGFP expression of untreated and dN-treated HLF cells transduced by wild type and Q151N vectors was analyzed by fluorescence microscopy (*A*) and FACS (*B*). The axis labels and gating procedures used were similar to those described above. The log copy number of HIV-1 2LTR DNA in dN treated or untreated HLFs at 6, 12, and 24 h post-transduction with WT or Q151N vector ($1.5 \times 10^6$ pg of p24 level) were determined by real time PCR. *BF*, bright field. *C*, * denotes no 2LTR copies.

cellular dNTP concentration, which is associated with the uncontrolled cell division phenotype of tumor cells, and a modified HIV-1 vector whose replication capability allows the vector to replicate efficiently only at high cellular dNTP concentrations. Numerous studies reported that dividing cells have higher dNTP concentrations than nondividing cells (10–12). In addition, tumor cells with a deregulated cell cycle and an accelerated replication cycle have higher cellular dNTP concentrations than normal dividing cells. The HIV-1 vector modification was achieved by using RT mutants with reduced dNTP binding affinity but with unaltered catalytic activity. Basically, the dNTP binding affinity of the RT variant in this modified HIV-1 vector is so low that this vector can replicate only in cells containing unusually high dNTP concentrations such as those found in tumors. We recently isolated and characterized an HIV-1 RT mutant, Q151N, which fulfills this kinetic and mechanistic requirement (18).

As shown in a series of experiments in this report, the Q151N HIV-1 vector can efficiently transduce several tumor cell lines, namely the A549 human lung epithelial tumor cell line and the PANC1 human pancreatic epithelial tumor cell line (Fig. 3). We also observed that the Q151N vector showed high gene delivery efficiency for two SV40 T antigen-transformed human cell lines, CHME5 and 293FT, containing high dNTP concentrations (Fig. 2*B*). However, the mutant HIV-1 vector

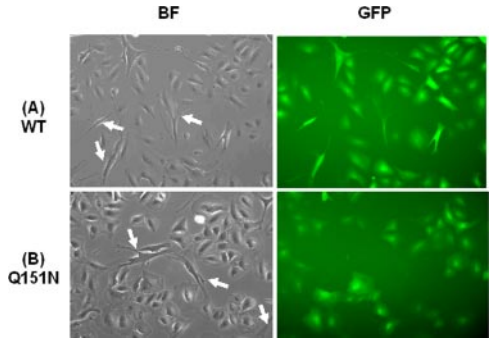



FIGURE 5. **Transduction of cocultured primary and tumor cells by wild type and Q151N vectors.** The cocultured primary HLFs and A549 human lung epithelial cells were transduced by an equal p24 level of wild type (*A*) and Gln-151 (*B*) HIV-1 vectors ($1.5 \times 10^6$ pg), and the EGFP expression in transduced cells was analyzed by fluorescence microscopy at 48 h post-transduction. *Arrows* indicate primary human lung fibroblasts.

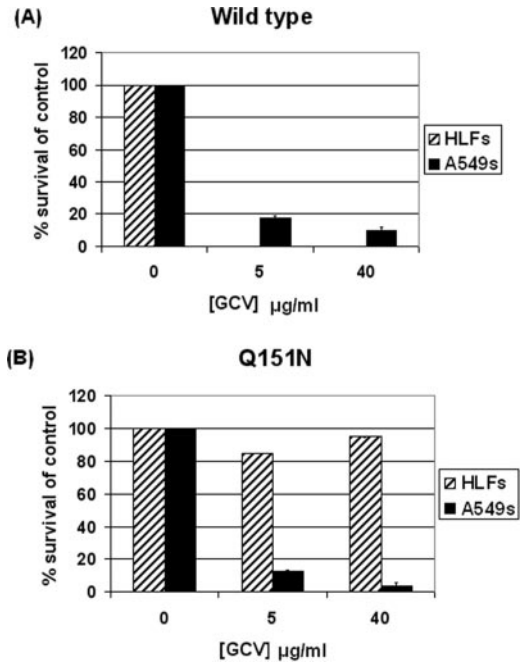



FIGURE 6. **Specific killing of tumor cells transduced by the HSV-TK-expressing Q151N vector by GCV.** HLF and A549 cells were transduced by an equal p24 level ($1.5 \times 10^6$ pg) of the wild type (*A*) or Q151N (*B*) HIV-1 vector expressing HSV-TK, and the transduced cells were treated with 5 and 40 $\mu$g/ml of GCV for 5 days, beginning 48 h post-transduction. The cells were counted after the 5-day GCV treatment, and the fraction of cells remaining after the GCV treatment was determined by comparing with untreated cells transduced with either the wild type or Q151N vector.

failed to transduce normal primary cells such as human lung fibroblasts and human foreskin keratinocytes. Unlike this mutant vector, however, the wild type HIV-1 vector transduced both normal and tumor cells used in this study with equal efficiency. Infections using wild type and mutant vectors at an equal p24 level showed very similar infectivity in CHME5 cells (Fig. 3*B*), suggesting that there is no significant infectivity difference between the wild type and mutant vectors in cells with high dNTP concentrations. This finding is consistent with biochemical observations that both wild type and mutant HIV-1 RTs showed similar high DNA polymerase activities at the high dNTP concentrations found in tumor cells (Fig. 1).

Our recent kinetic and structural studies suggested that the active site of Q151N mutant RT has lost the interaction with the 3′-OH group of the incoming dNTP substrate, reducing their binding affinity to dNTP substrates and consequently their dNTP incorporation efficiency, particularly at low dNTP concentrations (18). In contrast, wild type HIV-1 RT has a uniquely tight dNTP binding affinity, and this tight interaction with the 3′-OH group of dNTPs contributes to efficient DNA synthesis even at low dNTP concentrations. Therefore, these findings suggest that the HIV-1 RT interaction with the 3′-OH group of dNTP substrates is a key mechanistic element that contributes to the cell type specificity of HIV-1, which is also important in HIV-1 pathogenesis.

This study demonstrates two lines of evidence that the transduction failure of the Q151N mutant HIV-1 vector in normal primary cells is because of the absence of proviral DNA synthesis at the limited cellular dNTP concentrations found in normal primary cells. First, the pretreatment of human lung fibroblasts with dNs increased cellular dNTP concentrations by >200-fold compared with untreated cells and promoted the transduction of the Q151N mutant vector in primary human lung fibroblasts. Second, very restricted copy numbers of HIV-1 2LTR circle DNA, which is a molecular indicator for completion of the HIV-1 replication process, was observed upon the transduction of the mutant vector to primary lung fibroblasts. However, a significant increase of 2LTR circle DNA copy number was observed when dN-treated lung fibroblasts were transduced by the Q151N vector. These observations clearly support that poor dNTP availability was a rate-limiting factor in the transduction of primary lung fibroblasts by the mutant vector.

Preferential transduction by the Q151N vector to lung epithelial tumor cells was also observed in the coculture setting with normal lung fibroblasts. This experiment demonstrates a possible use of these modified HIV-1 vectors to specifically target tumor cells. Because the vectors used in this study are pseudotyped and only a single round of replication will occur upon transduction and because the Q151N RT is a high fidelity mutant (17), the RT contained in the mutant Q151N vector is highly unlikely to revert back to wild type RT. In addition, the model gene therapy system comprising the Q151N mutant vector expressing HSV-TK demonstrated that transduction by this modified vector renders GCV sensitivity preferentially to tumor cells with minimal cell death in primary human lung fibroblasts. Interestingly, because the HLF cells were not killed by the Q151N vector even at 5 days post-transduction, it is likely that the HSV-TK gene was not expressed in HLF cells transduced with the Q151N vector during the extended period of the GCV incubation. The pseudotyped vector used in this study also includes viral protein R (Vpr), which we demonstrated to be responsible for $G_2$ arrest (35). This $G_2$ arrest eventually leads to cell death of HIV-1-infected T cells. Therefore, it is possible that the vector used in this study can also lead to tumor-specific cell death even without the HSV-TK prodrug.

In conclusion, these results demonstrate that the modified vector system harboring the viral DNA polymerase mutant with reduced dNTP binding affinity can be a potential gene delivery system for the specific transduction of cells with high dNTP concentrations, such as tumor cells. This study suggests that the identification and use of unique cellular and virological factors essential for the specificity of viral based vectors can contribute to the development of safe and effective gene delivery tools.

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