Restriction Mapping of Retroviral Vector Episomal DNA

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The infidelity of reverse transcription enzyme activity during the replication cycle of a retrovirus can cause retroviruses to rapidly mutate. For gene therapy, this raises biosafety issues, such as the deletion of a therapeutic gene from a vector and the development of a replication-competent retrovirus. In a single retroviral replication cycle, a therapeutic herpes simplex virus thymidine kinase (HSVtk) gene was inactivated in approximately 8% of Moloney murine leukemia virus (MoMLV)-based vectors. In the same vector system, the mutation rates within a single retroviral vector were calculated as high as 3% per kb (9,14). In addition to the deletion of HSVtk from the retroviral vector, deletion mutations have been observed in retroviral vectors carrying various genes, including nerve growth factor receptor (7), α-rev sequence (2), human glucocerebrosidase (16) and luciferase (13). It is often difficult to distinguish a mutant vector from correct vector sequences in the genomic DNA of vector producer cells (VPC) because both vectors share large homologous DNA sequences except at the deletion or mutation site. To define the mutated region requires multiple Southern blots and analysis by restriction endonuclease mapping. This analysis can be complicated by interference with endogenous retroviral element sequences in the mammalian genome because these sequences are highly homologous to vector sequences.

High frequencies of superinfection and retrotransposition (manuscripts in preparation) of retroviral vector in cultured VPC results in detectable amounts of episomal DNA. Episomal DNA is advantageous for the Southern blot analysis of vectors because it is not subject to interference from endogenous retroviral sequences. Episomal vectors or retroviral sequences have been observed with other retroviruses, including mouse mammary tumor virus (11), avian sarcoma virus (15), avian leukemia virus (12), human immunodeficiency virus type 1 (HIV-1) (10) and in avian packaging cells (5). In this study, we successfully identified an HSVtk-deleted vector from VPC by analyzing episomal DNA directly instead of by genomic DNA restriction mapping. PCR primers were therefore designed accordingly to amplify this mutated region. The same primers were used to sequence this deletion without sequencing walking.

A LTKOSN.2 VPC was previously established in our group for a phase I human gene therapy clinic trial (4). pLTKOSN plasmid DNA (Figure 1A) was first introduced into the ecotropic packaging cell line GP+E86 (6) by transfection. Supernates from these cells were then used to transduce the amphotropic retroviral packaging line PA317 (8), which was selected in G418 (1 mg/mL) for two weeks. Twenty different VPC clones were isolated from the original pool of cells. LTKOSN.2 VPC produces viral titers of approximately 1 × 10^6 cfu/mL (4). A deletion of the HSVtk gene in LTKOSN.2 VPC was first detected in viral RNA collected from pelleted viral particles in Northern blot analysis by using different probes (Figure 1B). This result indicated that the titer calculated from only G418 resistant colonies did not represent the titer of the full-length LTKOSN vector, which implies that the evaluation of the vector titer needs to first ensure that all vectors contain an intact HSVtk suicide gene.

Unintegrated, episomal copies of viral DNA were used for Southern blot analysis to analyze this mutation without the interference with endogenous retroviral elements present in the cellular genomes. Small amounts of episomal DNA derived from vector sequences have been routinely detected within VPC from PA317 and GP+E86-derived VPC (unpublished data). Episomal DNA was extracted from both the cytoplasmic fraction and nuclear fraction of 1 × 10^7 LTKOSN.2 VPC. First, cells were trypsinized and subjected to 1% Triton® X-100 detergent for 5 min at room temperature to lyse the cellular but not the nuclear membrane. Nuclei were separated from the cytoplasmic fraction by centrifugation at 9500×g for 5 min at 4°C (3). Cytoplasmic fractions were subjected to phenol/chloroform extraction and ethanol precipitation to isolate purified episomal DNA. The episomal DNA in nuclei was extracted using Hirt’s method (1) with 5 M NaCl to remove genomic DNA. The supernate containing episomal DNA was isolated from cell nuclei by centrifugation (13 000×g for 15 min) and then subjected to phenol/chloroform extraction and ethanol precipitation. Episomal DNA samples extracted from both the cytoplasmic and nuclear fractions were evaluated by Southern blot analysis. The results clearly show that episomal DNA was mainly detected in
the cytoplasmic rather than the nuclear fraction of VPC (Figure 2).

To identify the primary structure of the deleted viral vector, restriction mapping and Southern blot analysis of the retroviral vectors were performed on the episomal DNA extracted from the cytoplasmic fraction of LTKOSN.2 VPC. The same membrane was hybridized at 42°C with various probes, including long terminal repeat (LTR; SacII/KpnI), extended packaging signal sequence (Ψ; SpeI/EcoRI), HSVtk (EcoRI fragment), simian virus 40 (SV40) promoter (BamHI/StuI) and Neo' (HindIII/BpmI), respectively (Figure 1A). Without restriction enzyme digestion, two respective sizes of episomal vector DNA were detected, 4.5 and 3.0 kb. Linear LTKOSN is represented by the 4.5 kb DNA band (Figure 3A, lane 1). BamHI digestion of the episomal DNA resulted in two fragments, 2.7 and 1.8 kb, generated from the 4.5 kb linear LTKOSN, while the second episomal proviral vector (3.0 kb) was resistant to BamHI digestion (Figure 3A, lane 2). This suggested that the 3.0 kb DNA (ΔLTKOSN) was a mutant of LTKOSN in which the BamHI site was deleted. The primary structure of this truncated 3.0 kb LTKOSN vector was found to include the 5′ LTR, extended packaging signal (Ψ and a portion of

Figure 1. Detection of a second mutated vector from LTKOSN.2 vector production. Viral RNA was extracted from virion particle pellet and subjected to Northern blot analysis. (A) Schematic diagram of LTKOSN vector and the probes. LTKOSN contains an HSVtk gene, which was cloned into the EcoRI site of LXSN. Extended packaging signal region (Ψ), neomycin phosphotransferase gene (Neo) and promoter sequence of SV40 early gene (SV). The probes were produced from the LTKOSN vector by the following restriction endonucleases: B, BamHI; Bp, BpmI; E, EcoRI; H, HindIII; K, KpnI; Sa, SacII; Sp, SpeI and St, StuI. (B) Viral supernatant was subjected to 20% sucrose gradient ultracentrifugation (125000×g) for 2 h at 4°C for virion pelleting and then extracted by RNAzol (Biotecx Laboratories, Houston, TX, USA). Viral RNA was subjected to Northern blot analysis on a 1% agarose 0.4 M formaldehyde gel. Two populations (4.5 and 3.0 kb) of vectors were detected from packaged viral transcripts by packaging signal probe (Ψ) and Neo' probe (Neo). Only one population (4.5 kb) of vector was detected by HSVtk probe (tk) probe.
the gag sequence), the Neo\(^{r}\) gene and the 3′ LTR, but did not contain the HSV\(tk\) gene and SV40 promoter. Using these restriction mapping results of retroviral episomal DNA, we designed a pair of primers that flanked the suspicious deletion region for PCR amplification. Sequencing was also performed using either of the PCR primers without further sequence walking. Extracted episomal DNA was first PCR amplified using a forward primer (5′-CTG TGT CTG TCC GAT TGT CTA GTG TC-3′) that was complementary to the extended packaging signal region, and a reverse primer (5′-CCC TTC CCG CTT CAG TGA CAA CG-3′) that was complementary to the Neo\(^{r}\) gene. The PCR included 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C and an extension for 2 min at 72°C. The PCR product was then purified by gel electrophoresis and subjected to sequencing analysis (DNA Sequencing Facility, Iowa State University, Ames, IA, USA). The deletion region in the mutant vector, ΔLTKOSN, included the entire HSV\(tk\) gene (except the EcoRI polylinker region at its 5′ end) and most of the 5′ end of SV40 promoter sequence (Figure 3B). Because only 81 bp of the 3′ end of the SV40 promoter remained adjacent to Neo\(^{r}\) gene, the 0.33 kb SV40 probe, which has only 52 bp overlap with this 81 bp, did not show any detectable signal for the ΔLTKOSN vector by episomal DNA Southern blot analysis (Figure 3). Therefore, Neo\(^{r}\) gene expression was driven by the 5′ LTR in ΔLTKOSN vector rather than the remaining truncated SV40 promoter sequences.

It has been demonstrated that recombinations of retroviral vector occur between two identical sequences at high frequency (62%) during reverse transcription (17). Therefore, ΔLTKOSN might arise from template switching be-

![Figure 2. Cellular location of episomal DNA in vector producer cells.](image)

![Figure 3. Characterization of mutated ΔLTKOSN by episomal DNA restriction mapping. (A) Episomal DNA was extracted from the cytoplasm fraction of LTKOSN.2 VPC with centrifugation after Triton X-100 detergent treatment to lyse the cellular but not the nuclear membrane (3). Episomal DNA extracted from cytoplasmic fraction was obtained using phenol/chloroform extraction and then ethanol precipitation. Episomal DNA extracted from nuclear fraction was obtained by adding 5 M NaCl to nuclei at 4°C overnight to precipitate genomic DNA. Supernatant of episomal DNA from the nuclear fraction was separated from genomic DNA pellet using centrifugation and then subjected to phenol/chloroform extraction (1). The vast majority of episomal DNA was detected in cytoplasmic fraction by Neo probe. Very little episomal DNA was detected in the nuclear fraction. Different salt concentration was used for the nuclear DNA extraction as reflected in the difference in band migration rate.](image)
tween two RNA molecules in the same virion when LTKOSN.2 VPC was established. The deletion of the HSV\textit{tk} gene from the LTKOSN vector appears to have occurred during construction of PA317 VPC because the ALTKOSN vector was not detected in LTKOSN-transfected GP+E86 VPC (manuscript in preparation). In conclusion, we demonstrate that the amount of retroviral episomal DNA obtained from the cytoplasm is sufficient for restriction mapping and can be used to define the deletion region of a retroviral vector. Following this method, which allows sequence analysis without interference from endogenous retroviral sequences present in host genomic DNA, primers can be designed to amplify this deletion region for sequencing.

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REFERENCES


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