Chimeric Retroviral Helper Virus and Picornavirus IRES Sequence
To Eliminate DNA Methylation for Improved
Retroviral Packaging Cells

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Our laboratory is interested in the genetic instability of retroviral vector producer cells (VPC) caused by host cell DNA methylation. We have observed that extensive DNA methylation can occur in murine LTKOSN.2 VPC of retroviral helper virus sequences at a rate of 2% of the population per day. The DNA methylation of the helper virus 5′ long terminal repeat (LTR) in LTKOSN.2 VPC correlated with reduced helper virus gene expression. These cells had significantly reduced Env-receptor interference and became target cells for vector reentry (superinfection). The VPC developed increasing genetic instability manifested by increasing vector copy numbers. The decreased helper virus gene expression, secondary to DNA methylation, dramatically reduced the vector titer of VPC (54). To overcome these limitations caused by host DNA methylation, we redesigned a retroviral helper virus to improve vector packaging efficiency and used this helper virus to study the interaction between host cells and retroviral sequences, especially host DNA methylation.

Mammalian DNA methyltransferase catalyzes the transfer of a methyl group to cytosines located 5′ to guanosine (CpG dinucleotide) and causes epigenetic effects which usually involve gene silencing. Methylated CpG dinucleotides inactivate gene expression by altering the DNA conformation (8, 22, 36) or attracting the binding of methylated CpG-binding proteins (13, 23, 38, 39) to impede transcription. The majority of DNA methylation patterns in mammalian genomes are found in retrovirus-related sequences, such as retrotransposons and endogenous or exogenous retroviruses (52). Evidence suggests that DNA methylation may act as a host defense system against retroviral invasion of the cellular genome (3, 51, 52). DNA methylation can be triggered by insertion of viral DNA sequence into chromosomes regardless of whether DNA transfection (2) or viral infection (16, 29, 45) was used to introduce the viral DNA sequences.

In several experimental systems, host cell methylation of retroviral provirus or retrotransposons has been evaluated. In a transgenic-mouse model, a retroviral provirus altered the methylation pattern within 1 kb of the retroviral integration site. The provirus was methylated, leading to an inactivation of transcription (17, 18). Sequences of small interspersed repetitive elements contained in the rat a-fetoprotein promoter region were associated with increased DNA methylation and decreased downstream reporter gene expression (12). Reduction of host DNA methylation leads to amplification and retrotransposition of kangaroo endogenous retroviral element 1 and xenologous recombination of chromosomes in interspecific mammalian hybrids of the Australian wallaby (41). Interestingly, retroviruses may benefit from host DNA methylation as well. Human immunodeficiency virus type 1 (HIV-1) infection may induce host DNA methylation activity, and as a consequence, the promoter region of gamma interferon was downregulated by DNA methylation (29). This may alter the balance of cytokines and reduce immune surveillance (28, 29).
The inactivation of HIV-1 or human T-cell leukemia virus type 1 gene expression by host DNA methylation of viral LTR regions may also induce latency of HIV-1 or human T-cell leukemia virus type 1 infection (1, 28, 44, 45).

These prior experiments did not evaluate DNA methylation of helper virus 5' LTR in VPC. Many commonly used retroviral vector packaging cell lines were established by cotransfection of two plasmids, one containing a helper virus genome and the other encoding a drug selection marker (26, 31–33). In this cotransfection system, selection for drug resistance does not provide active helper virus gene expression, and so the 5' LTR promoter region can be silenced by DNA methylation (54). Prior studies have demonstrated the concept of including an antibiotic selection marker (7) or a cell surface fluorescence-activated cell sorter marker (human Phoenix cell line; http://www.stanford.edu/group/nolan/ NL-Phoenix.html) downstream of gag-pol to monitor the gene expression. As reported here, a chimeric helper virus, pAM3-IRES-Zeo, was designed containing an internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus (19), a member of the picornaviruses (43), and a Zeocin resistance gene (Zeo) (10) to allow selection against DNA methylation that might occur in the helper virus 5' LTR region.

During translation of most eukaryotic mRNAs, ribosomes scan mRNA from the 5' cap sequence until an initiation codon is reached. In contrast, in picornavirus mRNA, ribosomes initiate translation by an alternative mechanism that involves internal initiation rather than scanning. The IRES sequences of picornavirus enable ribosomes to bind in a cap-independent fashion and start translation at the next AUG codon downstream (20). Ligation of the IRES sequence followed by Zeo at the 3' end of the env gene permits the translation of helper virus open reading frames and a selection marker from this mRNA (Fig. 1). Selection with Zeocin eliminates cells with methylated helper virus 5' LTR from the population. This design should ensure sustained helper virus gene expression, which would increase virion production and create sufficient Env receptor interference to prevent superinfection. The prevention of superinfection may in turn reduce replication-competent retrovirus (RCR) formation (34, 35). One additional advantage is that pAM3-IRES-Zeo allows the establishment of packaging cell lines within a shorter time. This advantage might be critical when making human VPC from a primary cell culture or stem cells to avoid immune rejection (48, 49), while transplantation of VPC into patients is necessary for continuous gene transfer (42).

**MATERIALS AND METHODS**

Construction of helper virus pAM3-IRES-Zeo and LEIN vector. An IRES sequence of encephalomyocarditis virus was isolated from the LXIN retroviral vector (Clontech, Palo Alto, Calif.) by NheI and PstI digestions and inserted into a PstI-linearized pZevoSV mammalian expression vector (Invitrogen, Carlsbad, Calif.) immediately 5' of the EM-7 prokaryotic promoter/Zeoicin resistance gene (Zeo) to create an IRES-Zeo expression cassette in plasmid pIRES-Zeo-SV40. Soft digestion of pIRES-Zeo-SV40 deleted the simian virus 40 (SV40) promoter and downstream polyadenylation signal to generate pIRES-Zeo. A 2.8-kb fragment consisting of the IRES-Zeo expression cassette, SV40 poly(A) signal, bacterial replication origin (ColE1 Ori), and phage replication origin (F1 Ori) was excised from pIRES-Zeo. The ColE1 Ori and ampicillin resistance gene (Amp') of pAM3 were replaced with the above 2.8-kb IRES-Zeo-containing fragment from pIRES-Zeo. The EM7 prokaryotic promoter located at the 5' end of the Zeo gene permits selection for pAM3-IRES-Zeo in bacteria. (B) Genomic RNA of MoMLV contains two internal stop codons at the 3' ends of the gag and pol genes that terminate cap-dependent translation and allow appropriate ratios of viral structural proteins. In pAM3-IRES-Zeo-derived transcripts, ribosomes also recognize the IRES sequence and initiate translation from the first AUG codon downstream of the IRES sequence. A portion of genomic RNA is spliced into env transcripts that are translated in a cap-independent mechanism. SD, splicing donor; SA, splicing acceptor.

**Cell culture and transfection.** Cell cultures were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technology Co.) plus 10% fetal calf serum under 5% CO2 at 37°C. The subclones of LTKOSN.2 VPC were obtained by limiting dilution of parental LTKOSN.2 VPC onto two 96-well plates (54). Helper virus and vector gene expression, DNA methylation status, and vector production in these subclones have been previously characterized (54). To rescue LTKOSN and ΔLTKOSN vectors from preexisting LTKOSN VPC subclones with methylated and silenced helper virus DNA, the subclones were transfected with pAM3-IRES-Zeo using FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, Ind.). To study the effects of host DNA methylation on retroviral helper virus without interference from chromosomal copies of pPAM3 present in LTKOSN VPC, pAM3-IRES-Zeo plasmid was phosphorylated as described.
transfected into NIH 3T3 tk− cells (ATCC CRL1658) utilizing Fugene 6 transfection reagent. A mixed population of pAM3-IRES-Zeo-transfected NIH 3T3 tk− cells, termed AMIZ cells, was established. Prior to transfection, pAM3-IRES-Zeo plasmid was linearized by XhoI digestion and 6 to 10 μg of pAM3-IRES-Zeo was then transfected to each well in six-well plates. Selection with Zeocin (350 μg/ml; Invitrogen) began 48 h after transfection and continued for at least 2 weeks. Transfection of the LEIN vector into the AMIZ cell pool and the CRL1619 (human melanoma) and IGROV cells (human ovarian carcinoma) (50), which were plated at 105 cells/well in six-well plates with 10 μg of protamine sulfate per ml. At 24 h after transfection, cells were selected for 10 to 14 days in medium containing G418 (1 mg/ml). Titers were calculated by multiplying the number of resistant colonies by the dilution factor.

To perform superinfection assays on AMIZ cells, supernatants containing LEIN vector collected from LEIN-transfected AMIZ cells were passed through a 0.45-μm pore-size syringe filter and diluted 10-fold and 100-fold before being used in superinfection assays. Along with AMIZ cells, NIH 3T3 tk− and PA317 cells were transfected as Env receptor interference-negative and -positive controls, respectively. Selection with G418 (1 mg/ml) on these transduced cells started 24 h after a single exposure to LEIN vector and continued for 10 to 14 days. The number of G418-resistant colonies was used as the index for superinfection on PA317 and AMIZ cells. To investigate the vector production capability of AMIZ cells, a LEIN vector from the ecotropic Moloney murine leukemia virus (MoMLV) packaging cell line, GP+E86, was transfected into AMIZ cells without further subcloning.

RNA analysis of helper virus and vector gene expression. Total cellular RNA was isolated from transfected cells and VPC by using the RNAeasy kit (Qiagen Inc., Valencia, Calif.) and Northern blotted from a 1% agarose–0.4 M formamide gel. Helper virus transcripts were detected with a Neo probe. Helper virus transcripts were detected by a 1.4-kb env probe, which was isolated from pAM3 after XhoI digestion. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to demonstrate similar RNA loading and to standardize the helper virus gene expression to allow comparisons between selected and unselected cells for analysis, the band intensities of both unspliced and spliced helper virus transcripts were divided by the intensity of GAPDH to determine relative expression levels.

DNA methylation analysis. In AMIZ cells transfected with LEIN vector, the methylation status of provirus and vectors was determined by evaluating the resistance to digestion with a DNA methylation-sensitive restriction endonuclease, SmalI, in the 5′ LTR region. Genomic DNA was digested with DraI and EcoRV to reduce the DNA fragment size, precipitated with ethanol, and then resuspended in sterile water. This DNA digest was divided into two equal portions, one of which was subjected to SmalI digestion. The Southern blot membrane was hybridized with a 428-bp fragment of the gag sequence (Pvull-DraI) from pPAM3 to detect helper virus and a GFP probe to detect the LEIN vector. The evaluation of these pAM3-IRES-Zeo-transfected cells demonstrated fairly equivalent RNA loading.

Without the interference of vector and endogenous retroviral sequences mentioned above, the DNA methylation status of the 5′ LTR region of pAM3-IRES-Zeo in AMIZ cells was determined by digesting genomic DNA with EcoRV, BstBI, and SmalI. If methylation occurred at the SmalI site, a 608-bp fragment would be excised instead of a 348-bp fragment when the DNA was probed with a 261-bp fragment excised from pAM3-IRES-Zeo by KpnI and AffI digestions. The degree of DNA methylation was calculated as the intensity of the SmalI-insensitive band divided by the sum of the intensities of this 608-bp fragment and the SmalI-sensitive fragment (348 bp) (see Fig. 5).

RESULTS

Construction of a chimeric retroviral helper virus with IRES and selection marker to allow direct selection of helper virus gene expression. We previously determined that DNA methylation occurred in 2% of the cell population per day within the 5′ LTR region of helper virus to inactivate helper virus gene expression in VPC (54). To eliminate methylated helper virus 5′ LTR from the packaging-cell population, a chimeric retroviral helper virus, pAM3-IRES-Zeo (Fig. 1), was constructed. The pAM3-IRES-Zeo construction allows Zeocin selection of cells with 5′ LTR promoter function, since helper virus and Zeo′ gene expression are transcribed from the 5′ LTR promoter (Fig. 1B). The selection with Zeocin maintains cells that also express helper virus and therefore counteract DNA methylation effects. Packaging cells based on this pAM3-IRES-Zeo helper virus should maintain high-titer production. The evaluation of these pAM3-IRES-Zeo-transfected cells with or without Zeocin selection provide a methylation profile of helper virus 5′ LTR and helper virus gene expression.

Analysis of chimeric pAM3-IRES-Zeo vector packaging ability in preexisting LTKOSN.2 VPC subclones. To test the packaging ability of pAM3-IRES-Zeo helper virus, pAM3-IRES-Zeo was transfected into three individual subclones of LTKOSN.2 VPC. LTKOSN.2 VPC contain one LTKOSN vector and an additional ΔLTOKSN, which is derived from the LTKOSN vector with a herpes simplex virus tk deletion mutation (53). The pPAM3 helper virus gene expression in these three LTKOSN.2 VPC subclones, 1, 3, and 5 (Fig. 2A, lanes 4 to 6), was inactivated by DNA methylation with impeded vector production ability (Table 1) (54). However, the LTKOSN (4.0-kb) and ΔLTOKSN (2.8-kb) vectors in these subclones were still transcribed (Fig. 2B, lanes 4 to 6) and no significant DNA methylation of these vectors was observed (54). This indicated that a key limitation of vector production in LTKOSN.2 VPC subclones is the lack of helper virus gene
IRES-Zeo was transfected into NIH 3T3 packaging cells without chromosomal pPAM3 effects, pAM3-positional interference. To establish a pooled population of 9 transfected cells would be required to study the DNA methylation in mammalian cells is site dependent within the genome (14). Therefore, a mixed population of pAM3-IRES-Zeo-transfected subclones, which indicates that the integration of pAM3-IRES-Zeo should be intact in transfected cells after selection. In contrast, cotransfection of pPAM3 without direct selection for pPAM3 gene expression but other selection markers in trans could result in randomly interrupted pPAM3 for integration. This was shown by two additional transcripts of lower molecular weight detected in PA317 and LTKOSN2.VPC (Fig. 2A). In addition to env transcripts, only one population of unspliced helper virus (gag-pol-env-IRES-Zeo) was detected in pAM3-IRES-Zeo-transfected subclones, which indicates that enhanced and sustained helper virus gene expression can be obtained in polyclonal packaging cells when pAM3-IRES-Zeo is used to allow Zeocin selection without the need to perform time-consuming cell subcloning. This implies a potential use of pAM3-IRES-Zeo to establish new packaging cells from other cells such as human primary cells.

Cells transfected with pAM3-IRES-Zeo provide a model to study DNA methylation of retroviral sequences. DNA methylation in mammalian cells is site dependent within the genome (14). Therefore, a mixed population of pAM3-IRES-Zeo-transfected cells would be required to study the DNA methylation of helper virus 5’ LTR in order to minimize the effects of positional interference. To establish a pooled population of packaging cells without chromosomal pPAM3 effects, pAM3-IRES-Zeo was transfected into NIH 3T3 tk− cells, and this was followed by selection with Zeocin without further subcloning. This pool of newly established packaging cells was named AMIZ packaging cells (pAM- IRES-Zeo). To allow DNA methylation to occur, AMIZ cells were released from Zeocin selection for 1 month and then placed in continuous culture with or without Zeocin selection for 78 days (10 passages). DNA methylation and gene expression of pAM3-IRES-Zeo were examined at 15, 54, and 78 days after being released from selection. Over the first 54 days of the cell culture period, DNA methylation of the 5’ LTR increased from 8 to 19%, and by day 78 it reached 61% (Fig. 3). The DNA methylation rate of helper virus 5’ LTR averaged 0.7% of the population per day during a 78-day period. AMIZ cells with continued Zeocin selection did not exhibit any detectable DNA methylation (Fig. 3). This drug selection effectively eliminated methylated pAM3-IRES-Zeo from the pooled AMIZ population.

Retroviral superinfection is blocked by enhanced helper virus gene expression. The effect of Zeocin selection on AMIZ cells was analyzed by gene expression of pAM3-IRES-Zeo in AMIZ cells. Gene expression of pAM3-IRES-Zeo in AMIZ cells with constant Zeocin selection showed a twofold increase compared to AMIZ cells without selection on day 15 and at least a fourfold increase on days 54 and 78 (Fig. 4). In contrast, pAM3-IRES-Zeo gene expression in AMIZ cells without Zeocin selection declined over time (Fig. 4, lanes 3, 5, and 7). Continuous Zeocin selection may have selected integration sites that are highly transcriptionally active and have less DNA methylation activity (5, 22).

We directly determined whether decreased pAM3-IRES-Zeo gene expression reduced Env receptor interference and increased vector superinfection. The susceptibility to superinfection was measured by exposing AMIZ cells from the above experiment to amphotropic LEIN vector supernatants and subjecting them to G418 selection. The number of G418-resistant colonies obtained from AMIZ cells with continued Zeocin selection was reduced from 23 on day 15 to no superinfection observed on days 54 and 78 (Table 2). In contrast, G418-resistant colonies obtained from AMIZ cells without Zeocin selection ranged from 1.2 × 105 to 5.6 × 106. These results demonstrate that increased gene expression of helper virus correlates with reduced susceptibility to superinfection.

### Table 1. Titer of LTKOSN.2 VPC subclones before and after transfection of pAM3-IRES-Zeo helper virus

<table>
<thead>
<tr>
<th>Subclone</th>
<th>pAM3-IRES-Zeo</th>
<th>Titer of subclone on target cells (CFU/ml)</th>
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<tr>
<td></td>
<td></td>
<td>NIH 3T3</td>
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<tr>
<td>1</td>
<td>+</td>
<td>1.1 × 10^7</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1 × 10^4</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>5 × 10^6</td>
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<tr>
<td></td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>1.6 × 10^7</td>
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<td>0</td>
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</table>

FIG. 3. DNA methylation of helper virus 5’ LTR over time with and without Zeocin selection. (A) Schema of the pAM3-IRES-Zeo helper virus, showing the restriction enzyme sites and the probe used for the methylation analysis. B, BstEII; E, EcoRV; S, Smal; AAA, SV40 polyadenylation signal. The drawing is not to scale. (B) A genomic DNA Southern blot membrane was probed with a 261-bp fragment excised from pAM3-IRES-Zeo with KpnI and AflII digestion. If methylation was present at the Smal site, a 608-bp fragment would result instead of a 346-bp fragment. The degree of DNA methylation was calculated as the intensity of the Smal-insensitive band (608 bp) divided by the sum of the intensities of this 608-bp band and the Smal-sensitive fragment (348 bp).
A high level of vector production is maintained by Zeocin selection. Vector production was analyzed in this AMIZ cell pool by transfecting LEIN vector into AMIZ cells and performing G418 selection to establish a VPC for titer assay. Zeocin selection was withdrawn from the AMIZ cell culture during the first 3 weeks of G418 selection after transfection with the LEIN vector. The titer obtained from this newly established uncloned population of AMIZ cells was $3.5 \times 10^6$ CFU/ml, which is 100-fold higher than the titer observed from a mixed population of PA317 cells transfected with LEIN vector ($4 \times 10^4$ CFU/ml). In addition, AMIZ cells were transduced with LEIN vector collected from LEIN-transfected GP+E86 cells, and an improved titer of $9 \times 10^6$ CFU/ml was obtained from a mixed cell population. To investigate whether selection with both Zeocin and G418 would adversely affect vector production, LEIN-transfected AMIZ cells were evaluated 56 (8 passages) and 67 (10 passages) days after transfection. Titers obtained from AMIZ cells transfected with LEIN ($3.5 \times 10^6$ CFU/ml on day 0) and placed under continuous selection with Zeocin and G418 were $2 \times 10^6$ CFU/ml (day 56) and $1.5 \times 10^6$ CFU/ml (day 67). In contrast, titers obtained from the same AMIZ cells transfected with LEIN but not subjected to G418 and Zeocin selection were only $2 \times 10^4$ and $4 \times 10^4$ CFU/ml on days 56 and 67, respectively. The reduced titer correlated with a significant decrease of both helper virus and vector gene expression when time points with and without selection were compared (Fig. 5). No significant increase of titer or helper virus gene expression was observed when the 17% DNA methylation present on day 0 was further reduced to 0% by day 56 after selection. This suggests a threshold effect, as we previously observed in cloned VPC (54). Substantial decreases of vector production, helper virus gene expression, and Env receptor interference were observed only when at least 60% methylation of the helper virus 5’ LTR occurred.

The DNA methylation status of 5’ LTRs of helper virus and vector were significantly increased in AMIZ cells transfected with LEIN vector and cultured without either G418 or Zeocin selection (Fig. 6). This increased methylation corresponded to the above-mentioned decreased vector titer and significantly reduced the gene expression of the helper virus and vector (Fig. 5). The DNA methylation of the helper virus 5’ LTR increased from 17% on day 0 to 30 and 36% on days 56 and 67, respectively. The average DNA methylation rate of helper virus 5’ LTR in AMIZ cells transfected with LEIN was estimated to be only 0.3% of the cell population per day during 67 days of continuous cell culture. In contrast, DNA methylation was not detected in AMIZ cells transfected with LEIN vector and placed under continuous G418 and Zeocin selection. No detectable DNA methylation occurred in the LEIN vector on day 0 (Fig. 6C, lanes 3 and 4), while the 5’ LTR helper virus showed 17% DNA methylation (Fig. 6B, lanes 3 and 4). This may be secondary to the timing of G418 and Zeocin selection. AMIZ cells transfected with LEIN vector were placed under G418 selection for 3 weeks to select for a LEIN-positive pop-

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**TABLE 2. Increased resistance of superinfection by Zeocin selection**

<table>
<thead>
<tr>
<th>No. of G418-resistant colonies on target cells</th>
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<tr>
<td>Day</td>
<td>NIH 3T3</td>
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<tr>
<td>-----</td>
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</tr>
<tr>
<td></td>
<td>No selection</td>
</tr>
<tr>
<td>15</td>
<td>$7 \times 10^4$</td>
</tr>
<tr>
<td>54</td>
<td>$6 \times 10^4$</td>
</tr>
<tr>
<td>78</td>
<td>$5 \times 10^4$</td>
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* Number of days after AMIZ cells were cultured in parallel either with or without Zeocin.
ulation, and Zeocin selection was not applied until day 0 in the experiment.

**DISCUSSION**

The experimental model described has demonstrated an approach using a retroviral helper virus combining a picornavirus IRES sequence and a selection marker gene that allows efficient elimination of methylated helper virus from packaging-cell populations. This strategy of using drug selection maintained high levels of helper virus gene expression and high-titer vector production ($1.5 \times 10^7$ CFU/ml) from a nonsubcloned population of VPC. The presence of greater Env receptor interference blocks vector superinfection and may reduce other potential problems with retroviral vectors, including replication-competent retrovirus formation and multiple copies of vectors. A new packaging cell pool, AMIZ cells, established by transfection of pAM3-IRES-Zeo chimeric helper virus into NIH 3T3 tk cells without any subcloning procedure, has proved a useful system to study the effect of host DNA methylation on retroviral sequences.

The selection of transfected cells (AMIZ cells) with Zeocin to maintain pAM3-IRES-Zeo gene expression eliminated DNA methylation from AMIZ cells and may also select cells with pAM3-IRES-Zeo helper virus integrated in optimal and active chromosomal regions. Ratios of pAM3-IRES-Zeo gene expression in selected AMIZ cells compared to nonselected AMIZ cells were about 2:1 on day 15 and at least 4:1 on days...
54 and 78 (Fig. 4), while helper virus showed only 12, 19, and 61% DNA methylation, respectively (Fig. 3). Similar results were also observed in AMIZ cells transfected with LEIN vector. Cells under continuous selection showed no detectable DNA methylation of the 5' LTR, but 30% (day 56) and 36% (day 67) DNA methylation was detected in cells without selection (Fig. 6). LEIN-transfected AMIZ cells under continuous selection had a vector titer of 1.5 x 10^7 CFU/ml on day 67, compared to 4 x 10^6 CFU/ml on day 67, in cells without selection. This, 1,000-fold difference in titer probably reflects the fact that structural proteins of viruses function as multimers (15). The formation of multimers occurs in a sigmoid rather than a linear dose-response fashion with respect to protein concentration that correlates more directly with helper virus gene expression and DNA methylation. The effect of host DNA methylation on the helper virus 5' LTR is therefore amplified by transcription, viral assembly, and then vector production.

To maintain efficient Env receptor interference and active viral production, a threshold level of helper virus gene expression is required. In retrovirus infection, this threshold level of gene expression is established by the accumulation of a sufficient copy number of virus through superinfection until efficient Env receptor interference is achieved and maintained (40). In our study, the threshold level of helper virus gene expression was achieved by Zeocin selection rather than by increasing the copy number of helper virus. Superinfection was observed when selection pressure was released and helper virus gene expression declined. These results support the conclusion that continuous selection of helper virus in VPC might enhance Env receptor interference and reduce the possibility of RCR formation.

For continuous virus production, retroviral gene expression has to be regulated at a sufficient level without interfering with host cell growth and differentiation. Increased levels of viral RNAs and proteins in infected cells can cause cytopathic effects, usually at the cost of cell death, by interrupting the production or translation of host mRNA (47). Although we observed that AMIZ cells under continuous selection did proliferate more slowly than AMIZ cells without selection, AMIZ cells under continuous G418 and Zeocin selection for high gene expression for 67 days (Fig. 5) still proliferated (data not shown). We did not attempt to select for pPAM3 gene expression by drug selection against the herpes simplex virus, shown). We did not attempt to select for pPAM3 gene expression declined. These results support the conclusion that continuous selection of helper virus in VPC might enhance Env receptor interference and reduce the possibility of RCR formation.

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REFERENCES