

# Stable Integration of Transgenes Delivered by a Retrotransposon–Adenovirus Hybrid Vector

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## ABSTRACT

Helper-dependent adenoviruses show great promise as gene delivery vectors. However, because they do not integrate into the host chromosome, transgene expression cannot be maintained indefinitely. To overcome these limitations, we have inserted an L1 retrotransposon/transgene element into a helper-dependent adenovirus to create a novel chimeric gene delivery vector. Efficient adenovirus-mediated delivery of the L1 element into cultured human cells results in subsequent retrotransposition and stable integration of the transgene. L1 retrotransposition frequency was found to correlate with increasing multiplicity of infection by the chimeric vector, and further retrotransposition from newly integrated elements was not observed on prolonged culture. Therefore, this vector, which utilizes components of low immunogenic potential, represents a novel two-stage gene delivery system capable of achieving high titers via the initial helper-dependent adenovirus stage and permanent transgene integration via the retrotransposition stage.

## OVERVIEW SUMMARY

A retrotransposition-competent LINE-1 element (RC-L1) was inserted into a helper-dependent adenovirus (HDAd) to generate a hybrid adenovirus–retrotransposon vector. The hybrid vector could be amplified to high titer and was capable of efficiently infecting a variety of human cells as an adenovirus. The RC-L1 within the hybrid vector then underwent retrotransposition in target cells, resulting in stable integration of the linked transgene cassette. A linear increase in retrotransposition frequency was observed when cells were infected at increasing multiplicities of infection. Thus, we have developed a novel hybrid vector that can achieve efficient transduction via the HDAd stage and stable integration of transgenes via the RC-L1 stage.

## INTRODUCTION

THE LACK OF AN OPTIMAL METHOD for direct gene transfer and permanent transduction of tissues *in vivo* is a significant impediment to the success of human gene therapy. In seek-

ing to achieve efficient gene transfer, a variety of viruses have been adapted for use as gene delivery vectors, including retrovirus, adenovirus, and adeno-associated virus. Of these, adenoviral vectors are capable of achieving high titers and have been shown to efficiently infect many cell types *in vivo* by direct injection. However, because the adenovirus life cycle is inherently episomal in nature, and lacks the machinery for efficient integration into host chromosomes, adenoviral integration events are rare (Harui *et al.*, 1999), and transgene expression is transient in duration. Moreover, the utility of adenovirus vectors is further limited by cellular and humoral immune responses against the adenoviral gene products, which can be expressed at low levels in the transduced cells despite deletion of the E1 regulatory region (Yang *et al.*, 1996a,b; Kaplan *et al.*, 1997; Sparer *et al.*, 1997).

As one approach to overcome these limitations, helper-dependent adenoviral vector systems, which lack all the coding sequences that could be toxic or immunogenic to the host (Mitani *et al.*, 1995; Kochanek *et al.*, 1996; Scheidner *et al.*, 1998), have been developed. These modifications not only allow an expanded cloning capacity with the ability to insert up to 38 kb of foreign DNA into the vector, but also result in prolonged ex-

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pression of the transgene compared with standard E1-deleted adenovirus vectors (Morrall *et al.*, 1998, 1999; Morsy *et al.*, 1998; Scheidner *et al.*, 1998), presumably because host immune responses against the vector are reduced. Nevertheless, it is unlikely that the transferred genes would persist permanently.

Human retrotransposons (i.e., long interspersed nuclear elements; LINE-1s or L1s) also have the potential to serve as gene delivery vectors. Retrotransposition-competent L1s (RC-L1s) are 6.0 kb and contain a 5' untranslated region (5' UTR) harboring an internal promoter (Swergold, 1990; Minakami *et al.*, 1992), two nonoverlapping open reading frames (ORF 1 and ORF 2), and a 3' UTR that ends in a polyadenylic acid tail. ORF 1 encodes a 40-kDa nucleic acid-binding protein that displays nucleic acid chaperone activity *in vitro* (Holmes *et al.*, 1992; Hohjoh and Singer, 1996; Martin and Bushman, 2001). ORF 2 encodes a protein with reverse transcriptase (RT) and endonuclease (EN) activities (Feng *et al.*, 1996). Transgene cassettes can be inserted into the 3' UTR of RC-L1s, and the resultant constructs can retrotranspose from an extrachromosomal self-replicating plasmid episome into genomic DNA at a high frequency, resulting in stable integration of the transgene (Moran *et al.*, 1996). However, a rate-limiting step in further developing L1s as an integrating vector system has been their inefficient delivery to target cells.

Here we describe a novel hybrid vector, consisting of an RC-L1 encoded by a helper-dependent adenovirus vector. After initial transduction of cultured human cells by the adenovirus carrier, expression of the L1 and its subsequent retrotransposition result in stable integration of the transgene cassette. Thus, this L1-adenovirus hybrid vector simultaneously overcomes the problems of transient adenovirus-mediated transgene expression and low efficiency of retrotransposon vector delivery. It represents a novel vector system that is capable of achieving high-titer gene transfer as well as stable integration and permanent transduction.

## MATERIALS AND METHODS

### Cells and plasmids

Human embryonic kidney (HEK) 293A cells (Quantum Biotechnologies, Laval, Quebec, Canada); 293Cre4 cells, which stably express the Cre recombinase (Chen *et al.*, 1996) (Merck, Rahway, NJ); HeLa human cervical carcinoma cells (American Type Culture Collection [ATCC], Rockville, MD); and CFBE41<sup>0-</sup> immortalized human bronchial epithelial cells (generously provided by D. Gruenert, University of California at San Francisco, San Francisco, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (20 U/ml), and 0.4 mM L-glutamine (DMEM-complete). 293Cre4 cells were cultured in the presence of G418 (400 µg/ml). Plasmid pSTK120, which contains genomic hypoxanthine phosphoribotransferase (HPRT) and cosmid C346 stuffer sequences flanked by adenovirus serotype 5 (Ad5) inverted terminal repeats and packaging signal (Kochanek *et al.*, 1996), was generously provided by S. Kochanek (University of Cologne, Cologne, Germany). The construction of plasmid pJM130, which contains the human retrotransposon L1.3 (Dombroski *et al.*, 1993) linked to a neomycin resistance transgene cassette (L1.3mneoI), has been

described previously (Sassaman *et al.*, 1997). Plasmids were purified with Qiagen (Valencia, CA) Maxiprep columns according to the manufacturer instructions.

### Construction of helper-dependent adenovirus-retrotransposon hybrid vector

A cytomegalovirus (CMV)-driven green fluorescent protein (GFP) marker gene cassette was inserted between *Bam*HI sites in plasmid pSTK120 to generate an intermediate helper-dependent adenovirus construct, designated pSMK-GFP. A *Sca*I-*Apa*I restriction fragment containing the CMV-driven L1.3mneoI sequence was then excised from pJM130 (Sassaman *et al.*, 1997), and ligated into the pSMK-GFP backbone after digestion with *Eco*RV and *Apa*I; this procedure also results in removal of the unstable *Alu* repeats in the HPRT intron sequence (Sandig *et al.*, 2000). The resultant plasmid, pSMK-L1.3mneoI, thus contains a 14.7-kb helper-dependent adenovirus (HDAd) vector construct that encodes two independent cassettes, CMV-L1.3mneoI and CMV-GFP.

**HDAd vector production.** The helper virus AdLC8cluc, a recombinant Ad5 vector containing *loxP* sites flanking the packaging signal, was used to propagate the helper-dependent vector in 293Cre4 cells (Chen *et al.*, 1996). After transfection of *Pme*I-linearized pSMK-L1.3mneoI, the resultant helper-dependent virus HDAd-L1.3mneoI was propagated by serial passage on 293Cre4 cells with addition of the AdLC8cluc helper virus at each stage, and purified by double cesium chloride gradient ultracentrifugation (Parks *et al.*, 1996). Helper virus contamination levels were determined by median tissue culture infective dose (TCID<sub>50</sub>) and plaque assays on 293A cells. The helper-dependent adenovirus was titered (transducing units, TU/ml) by flow cytometric analysis of GFP expression from cells infected with limiting dilutions of purified vector.

**Southern blot analysis of adenovirus DNA.** Approximately 10<sup>7</sup> TU of HDAd-L1.3mneoI virion DNA was incubated with 200 µl of capsid digestion buffer (50 mM Tris-HCl [pH 8.0], 1.0 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS], and proteinase K [1.0 mg/ml]) at 50°C for 1 hr (Fisher *et al.*, 1996a). Reactions were cooled to room temperature, extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated with ethanol, and the virion DNA was resuspended in 10 mM Tris-HCl, 0.1 mM EDTA. Virion DNA was digested with restriction enzymes *Bst*Z1107I (New England BioLabs, Beverly, MA), *Eco*RI (GIBCO, Rockville, MD), and *Not*I (New England BioLabs), fractionated on a 0.7% agarose gel, blotted, and probed with a <sup>32</sup>P-labeled *Pme*I restriction fragment from pSMK-L1.3mneoI (Decaprime II; Ambion, Austin, TX). Southern blots were analyzed by autoradiography or by PhosphorImager 840 (Molecular Dynamics, Sunnyvale, CA).

### Retrotransposition assay

HeLa cells (3–5 × 10<sup>5</sup>) were infected with serial dilutions of HDAd-L1.3mneoI (multiplicities of infection [MOIs] of 1, 10, and 100). On day 2 posttransduction, the infected HeLa cells were trypsinized and counted by hemocytometer, and cell dilutions were plated in duplicate for selection in DMEM-complete plus G418 (600 µg/ml). Uninfected HeLa cells were plated in parallel as a negative selection control. In addition, an aliquot of the

cells from each MOI was analyzed for GFP expression by flow cytometry at various time points. Medium was changed every 2–3 days with DMEM-complete plus G418 (600  $\mu\text{g/ml}$ ) for 14 days. The resultant G418-resistant (G418<sup>R</sup>) colonies were fixed and stained with 0.4% Giemsa for visualization. In addition, individual G418<sup>R</sup> clones from each MOI were harvested before fixation and expanded in DMEM-complete plus G418 (600  $\mu\text{g/ml}$ ), and genomic DNA was harvested by standard methods (Maniatis *et al.*, 1982). Retrotransposition frequency was determined as number of G418<sup>R</sup> colonies divided by number of GFP-positive cells, and normalized to HDAd-L1.3mneoI copy number per cell as quantitated by PhosphorImager analysis. Retrotransposition titer was determined as input adenovirus titer multiplied by retrotransposition frequency.

#### PCR analysis for retrotransposition

Polymerase chain reaction (PCR) was performed on genomic DNA from individual G418<sup>R</sup> colonies to determine whether proper splicing of the *neo*<sup>R</sup> transgene had occurred during retrotransposition. Each PCR was performed in a 50- $\mu\text{l}$  volume containing 10 U of *Taq* polymerase, 0.2 mM dNTPs, ~200 ng of genomic DNA template, and 200 ng of primers 437S and 1808S, which are specific for the *neo*<sup>R</sup> gene. PCR was performed on a PTC-100 thermocycler (MJ Research, Waltham, MA), using a cycling program of 94°C, 1 min (1 cycle); 94°C, 1 min; 64°C, 30 sec; 72°C, 1 min (30 cycles); and a final extension of 72°C for 10 min. One-fifth of the reaction volume was loaded onto a 1.25% agarose gel containing ethidium bromide.

#### Southern blot analysis of retrotransposition events

After adenoviral infection at increasing MOIs and G418 selection, 20  $\mu\text{g}$  of genomic DNA from individual G418<sup>R</sup> colonies was digested with *Eco*RI and fractionated on a 0.8% agarose gel containing ethidium bromide. The resolved DNA was transferred to a nylon membrane (Hybond-N+; Amersham, Arlington Heights, IL) and hybridized with a <sup>32</sup>P-labeled DNA probe to the *neo*<sup>R</sup> gene (Decaprime II; Ambion). To determine the copy number of HDAd-L1.3mneoI after transduction (Fig. 3A), total genomic DNA was harvested 24 hr posttransduction with serial MOIs, digested with *Sca*I, resolved on a 0.8% agarose gel, and subjected to Southern blot analysis with a <sup>32</sup>P-labeled DNA probe to the GFP gene. The standard curve was generated by addition of pSMK-L1.3mneoI plasmid DNA equivalent to 0.5 to 100 copies per cell (Park *et al.*, 2000) to genomic DNA from uninfected cells. Since the HDAd-L1.3mneoI vector genome contains two copies of the GFP marker gene, two molar equivalents of pSMK-L1.3mneoI was added to the uninfected genomic DNA to create the standard curve. Southern blots were analyzed by autoradiography or with a PhosphorImager 840 (Molecular Dynamics).

## RESULTS

#### Construction, propagation, and characterization of HDAd-L1.3mneoI hybrid vector

The basic structure of the L1-adenovirus hybrid vector construct (pSMK-L1.3mneoI) is shown in Fig. 1A. In this construct, a retrotransposon element (L1.3) linked to a neomycin

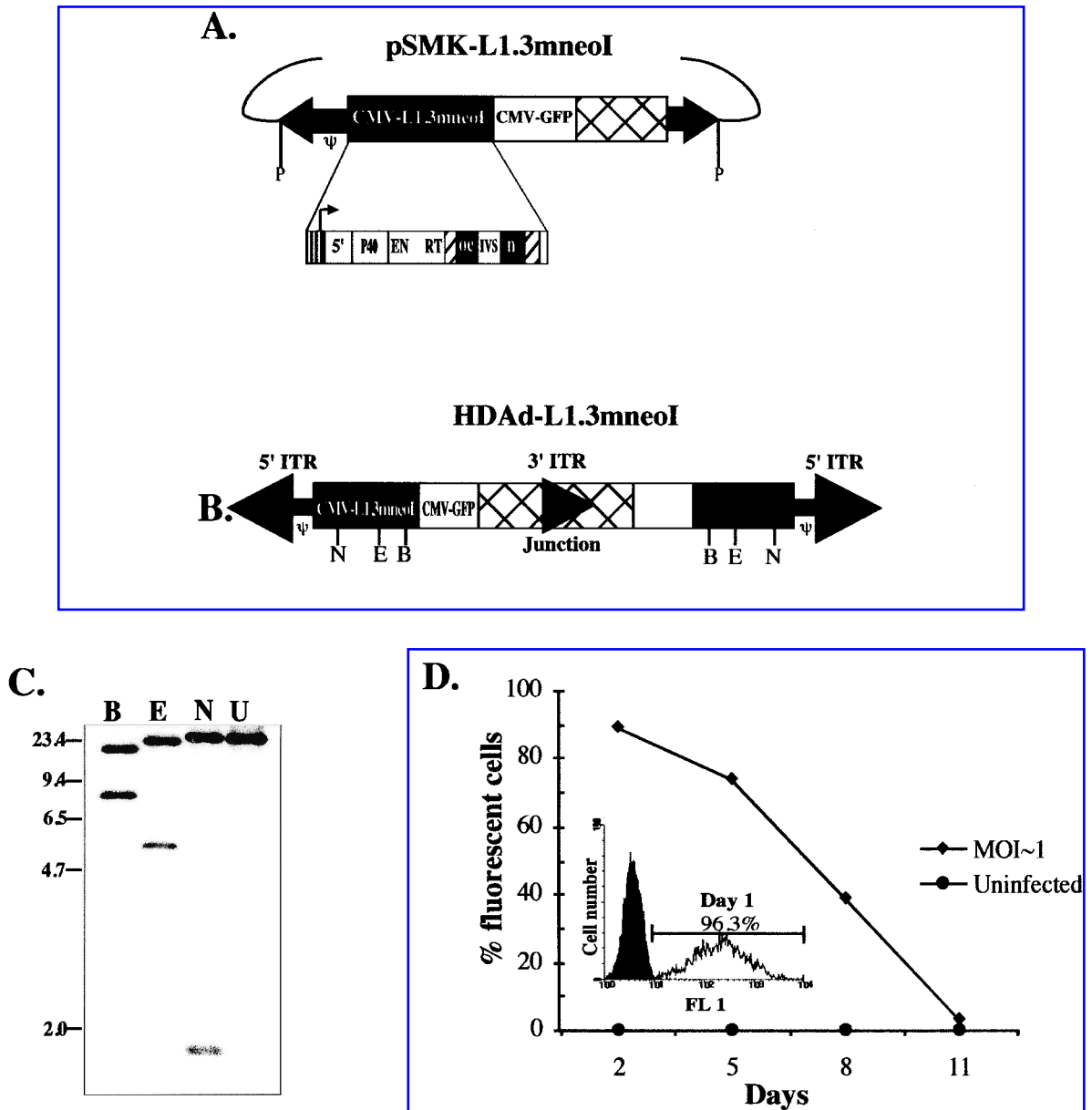
resistance (*neo*<sup>R</sup>) retrotransposition indicator transgene cassette (mneoI) was inserted into a 14.7-kb helper-dependent adenovirus construct. The *neo*<sup>R</sup> gene is inserted in the antisense orientation with respect to L1.3 and is disrupted by an intron (IVS 2 of the  $\gamma$ -globin gene), which is in the sense orientation (see also Fig. 2A). The arrangement of this retrotransposon/transgene construct (here designated L1.3mneoI) has been described previously (Moran *et al.*, 1996; Sassaman *et al.*, 1997), and ensures that only transgene expression linked to authentic retrotransposition events will be scored. A green fluorescent protein (GFP) marker gene driven by a cytomegalovirus (CMV) promoter is also contained within the backbone of the helper-dependent vector. As expression of the GFP marker gene is unlinked to the L1.3mneoI retroelement, it serves as an independent marker of adenoviral transduction and allows convenient determination of the helper-dependent adenovirus titer by flow cytometry. The only adenovirus-derived sequences contained within the chimeric vector are the inverted terminal repeats (ITRs) and the packaging signal sequence (*ψ*), which are required for its propagation (Kochanek *et al.*, 1996; Morsy *et al.*, 1998).

To propagate the helper-dependent vector derived from pSMK-L1.3mneoI, essential viral functions were provided in *trans* by AdLC8cluc, an attenuated helper virus that contains *loxP* sites flanking its packaging signal. Thus, when coamplified in a human 293-derived cell line expressing the Cre recombinase (293Cre4) (Chen *et al.*, 1996), the packaging signal is efficiently deleted from the helper virus, which inhibits its ability to propagate and thereby confers a growth advantage to the helper-dependent vector (Parks *et al.*, 1996). Purified helper-dependent adenovirus preparations were obtained at titers of  $4.74 \times 10^9$  transducing units (TU)/ml, as determined by flow cytometric analysis of GFP expression in human 293 and HeLa cells after transduction with serial dilutions of virus (Table 1). Levels of contamination by residual helper virus were less than 0.1% by TCID<sub>50</sub> assays (data not shown).

To determine the genomic structure of the retrotransposon-adenovirus hybrid vector, DNA was isolated from helper-dependent virions purified by double cesium gradient ultracentrifugation and analyzed by Southern blot (Fig. 1B and C). The results indicate that the helper-dependent adenovirus, designated HDAd-L1.3mneoI, consists of a concatemer of the original 14.7-kb pSMK-L1.3mneoI vector construct (Fig. 1B). This was not unexpected, since helper-dependent adenovirus vectors with smaller genomes can be packaged if concatemerization of the vector sequence occurs, resulting in a multimeric size that is within the packageable size range of 27 to 38 kb (Parks and Graham, 1997; Morsy *et al.*, 1998). The restriction digest patterns from Southern blot analysis of adenovirus DNA are consistent with the presence of only a single species of helper-dependent adenovirus derived from “tail-to-tail” concatemerization of the original hybrid vector construct (Fig. 1C).

#### Helper-dependent adenovirus stage of the hybrid vector mediates efficient but transient transduction of human cells

The HDAd-L1.3mneoI helper-dependent adenovirus is able to infect a number of different human cell lines, including 293A, HeLa, and CFBE41<sup>o7</sup>. Infections were initially performed at low multiplicities of infection (MOI of ~1) to ensure that pre-



**FIG. 1.** (A) Hybrid vector construct pSMK-L1.3mneoI. The sequences composing pSMK-L1.3mneoI are, from left to right, the 5' inverted terminal repeat (ITR) of Ad5 (nt 1–440) including the packaging signal ( $\psi$ ); the CMV-L1.3mneoI cassette from pJM130 (10,236 bp), a CMV-GFP marker cassette (1914 bp), C346 cosmid stuffer sequence (2038 bp, cross-hatched box) from pSTK120, and the Ad5 3' ITR (117 bp). *PmeI* restriction sites (P) flanking the ITRs release the 14.7-kb hybrid vector construct from the plasmid backbone before amplification in 293Cre4 cells. The organization of the CMV-L1.3mneoI retrotransposon element is shown in detail, and consists of a CMV promoter (vertically lined box), the 5' untranslated region (5'), ORF 1 product P40, ORF 2 product EN and RT, and the *neo<sup>R</sup>* transgene, which is cloned into the 3' untranslated region of L1.3 in the reverse orientation (oen) and flanked by SV40 promoter and polyadenylation sequences (hatched rectangles), and is interrupted by a forward-splicing intron (IVS). (B) The HDAd-L1.3mneoI helper-dependent adenovirus genome after virus rescue and amplification is a 29.4-kb tail-to-tail concatemer of the pSMK-L1.3mneoI hybrid vector construct. The restriction sites depicted are as follows: B, *Bst*1107I; E, *EcoRI*; N, *NotI*. (C) Southern blot of HDAd-L1.3mneoI virion DNA hybridized with a <sup>32</sup>P-labeled 14.7-kb *PmeI* fragment from pSMK-L1.3mneoI. The restriction digest patterns are consistent with a tail-to-tail concatemer. Lane B, *Bst*1107I digest (7875-bp doublet band and 13,740-bp single band); lane E, *EcoRI* digest (5336-bp doublet band and 18,818-bp single band); lane N, *NotI* digest (1904-bp doublet band and 25,682-bp single band); lane U, uncut HDAd-L1.3mneoI virion DNA (>23-kb marker).  $\lambda$ /*HindIII* ladder molecular weight values are depicted on the left. (D) The first-stage HDAd-L1.3mneoI adenovirus shows efficient but transient expression. HeLa cells infected with HDAd-L1.3mneoI (MOI of  $\sim$ 1) and analyzed by flow cytometry were 96.3% positive for GFP expression 24 hr posttransduction (*inset*: uninfected negative control cells are represented as the dark shaded area). Infected and uninfected HeLa cells were then serially passaged, and GFP expression was again determined on days 2, 5, 8, and 11 posttransduction.

TABLE 1. DETERMINATION OF RETROTRANSPOSITION FREQUENCIES AND TITERS<sup>a</sup>

MOI	HDAd-L1.3 mneo (copies/cell)	Input HDAd-L1.3 mneo titer (TU/ml)	Retrotransposition frequency	Retrotransposition titer (IU/ml)
1	0.8	$4.74 \times 10^9$	1:14,286	$3.33 (\pm 0.6) \times 10^5$
10	6	$4.74 \times 10^9$	1:1,382	$3.42 (\pm 0.4) \times 10^6$
100	65	$4.74 \times 10^9$	1:283	$1.68 (\pm 0.05) \times 10^7$

Abbreviations: MOI, Multiplicity of infection; TU/ml, transducing units per milliliter; IU/ml, integrating units per milliliter.

<sup>a</sup>The retrotransposition frequency was normalized to 1, 10, and 100 copies of HDAd-L1.3 mneoI per cell as determined by PhosphorImager analysis. The retrotransposition titer for each MOI was determined as (input HDAd-L1.3 mneoI titer)  $\times$  (normalized retrotransposition frequency); see text for details.

dominantly single-copy infections would occur. Under these conditions, GFP expression from HDAd-L1.3mneoI should follow the transient kinetics of low-copy adenovirus transduction. As expected, flow cytometric analysis of GFP expression in adenovirus-infected HeLa cells demonstrated highly efficient transduction initially (Fig. 1D, inset), with expression levels progressively diminishing over a period of several days as the cells were serially passaged (Fig. 1D, graph). Similar results were obtained from the other human cell lines tested (data not shown).

#### *Retrotransposon delivered by the hybrid vector is functional*

As described above, the *neo*<sup>R</sup> transgene cassette linked to the L1.3 element serves as an indicator of retrotransposition. Thus, G418 resistance will arise only if L1.3 is transcribed from HDAd-L1.3mneoI, the intron disrupting the *neo*<sup>R</sup> coding sequence is spliced out of the mRNA, and the mRNA is subsequently reverse transcribed and integrated into genomic DNA. The now intact *neo*<sup>R</sup> gene can then be expressed from the simian virus 40 (SV40) promoter (Moran *et al.*, 1996; Sassaman *et al.*, 1997) (see Fig. 2A for details). Only true retrotransposition events are scored in this assay, as mRNAs originating directly from the SV40 promoter in the original helper-dependent adenovirus cannot be spliced, and therefore an intact *neo*<sup>R</sup> gene product will not be produced. Consequently, even if rare adenoviral integration events were to occur, the cells would remain G418 sensitive unless a true retrotransposition/integration event occurred in the same cell.

The splicing required for reconstitution of the *neo*<sup>R</sup> transgene provides a convenient assay for the presence of authentic retrotransposition events, resulting in permanent integration into the target cell genome. Accordingly, HeLa cells were infected at increasing MOIs of HDAd-L1.3mneoI, the infected cells were placed in G418 selection 48 hr posttransduction, and selection was continued for 14 days. These G418-selected cells no longer exhibited GFP fluorescence, suggesting that the adenovirus episome had already been eliminated. Genomic DNA was isolated from more than 20 individual G418-resistant clones that had been expanded, and PCR analysis was performed to determine whether proper splicing of the intron had occurred before reverse transcription and genomic integration. Only the appropriately spliced PCR product was detected after amplification of genomic DNA from all (>20) G418-resistant colonies tested, regardless of the MOI used for initial infection (Fig. 2B). We did not detect the larger unspliced PCR product

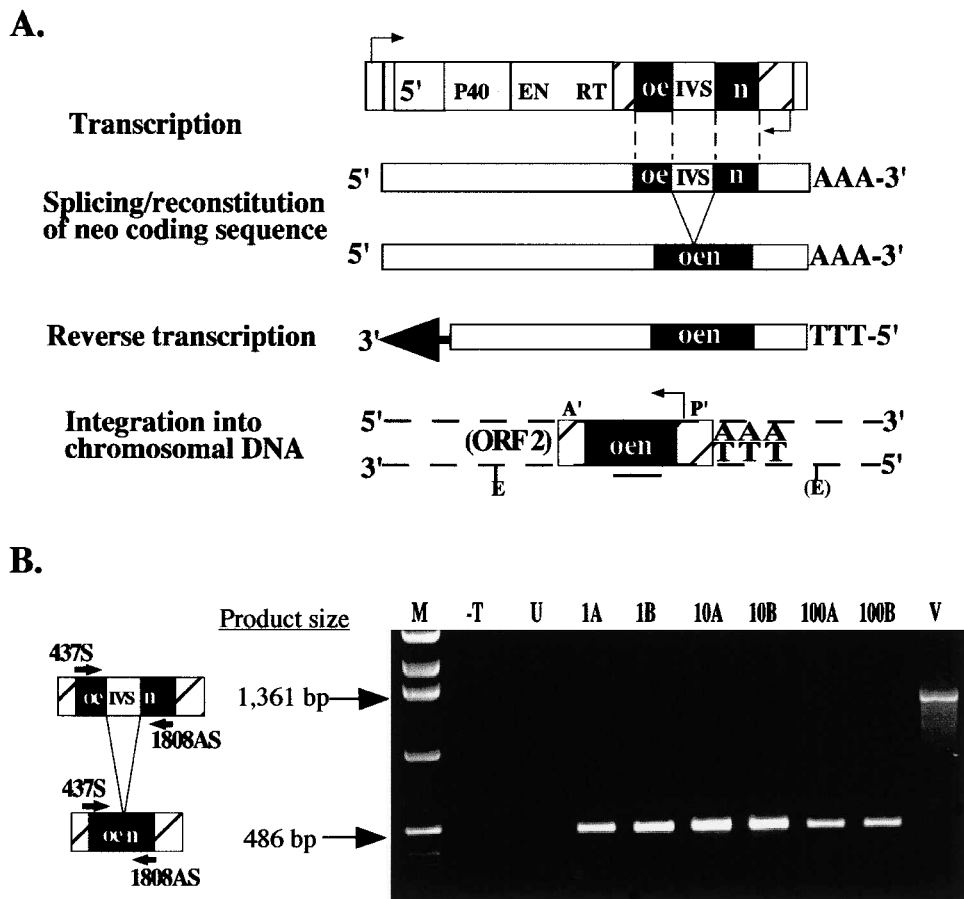
that would indicate the presence of the original adenovirus vector. Thus the G418-resistant phenotype was indeed the result of authentic L1.3mneoI retrotransposition events originating from the retrotransposon-adenovirus hybrid vector.

#### *Genomic Southern blot analysis of retrotransposon-mediated transgene integration events*

To further confirm that the *neo*<sup>R</sup> transgene did integrate into the genomic DNA and to examine the pattern of integration, we performed Southern blot analysis of genomic DNA isolated from G418-resistant HeLa clones that had been initially transduced at increasing MOIs. These genomic DNA samples were digested with *Eco*RI, which cuts only once within L1.3mneoI, and hybridized with a radiolabeled *neo*<sup>R</sup> probe (Fig. 2A). These clones were each originally derived from independent G418-resistant colonies originating from different plates within the same experiment or different experiments. Figure 3A shows Southern blot results from a representative set of G418-resistant HeLa clones. Only one major restriction fragment was detected by the *neo*<sup>R</sup> probe in the majority of G418-resistant clones examined, regardless of the initial MOI (Fig. 3A: MOI of 1, 1A–1D; MOI of 10, 10A–10D; MOI of 100, 100A–100D). The fragment sizes ranged from  $\sim 2.5$  to  $>12$  kb, suggesting the L1.3mneoI element had integrated into random genomic locations. Multiple integration signals were observed in only 1 of more than 20 independent clones examined, which had originally been transduced at an MOI of 100 (data not shown). Thus, although initial adenovirus-mediated delivery of up to 100 copies of the L1.3mneoI retroelement per cell correlates with an increase in the retrotransposition frequency in the overall population, it appears that generally only single-copy integration events occurred within each cell. Dose-dependent cytotoxicity was observed at MOIs greater than 100, preventing analysis of multiple integration events at high copy numbers.

#### *Previously integrated retrotransposon-transgene elements are not mobilized with prolonged culture*

To determine whether additional retrotransposition events would occur over time from the initially integrated L1.3/*neo* retroelements, three G418-resistant HeLa clones were serially passaged an additional seven times (passages 1–8) under G418 selection over a period of 4 weeks, and genomic DNA from passage 8 clones were isolated for Southern blot analysis as described above. In all three G418<sup>R</sup> clones analyzed, the same size fragment was detected in both passage 1 and passage 8, in-



**FIG. 2.** (A) Overview of the L1.3mneoI retrotransposition assay. The CMV promoter drives transcription of L1.3mneoI, but the *neo<sup>R</sup>* gene is in the antisense orientation and disrupted by an intron (IVS) in the sense orientation. Thus, a functional *neo<sup>R</sup>* gene product will not be obtained unless the IVS is spliced out of the RNA transcript, followed by reverse transcription and integration of the intact *neo<sup>R</sup>* transgene. Subsequent expression of the *neo<sup>R</sup>* gene product from its own promoter (P') ensures that G418 resistance is conferred only to cells that have undergone authentic L1-mediated retrotransposition. The *neo<sup>R</sup>* gene probe used for Southern blot analysis to examine the integration events occurring after retrotransposition from HDAd-L1.3mneoI was generated by [<sup>32</sup>P]dCTP random priming of a 460-bp *neo<sup>R</sup>* gene fragment, and is indicated by the bar. The single *EcoRI* site [(E)] present in L1.3 and the putative *EcoRI* site in genomic DNA [(E)] are also represented. (B) PCR assay for correct splicing of the artificial intron in the *neo<sup>R</sup>* gene after retrotransposition from the HDAd-L1.3mneoI hybrid vector. The schematic at left illustrates the unspliced product size and the correctly spliced product size after PCR amplification with *neo<sup>R</sup>*-specific primers. Genomic DNA from individual G418<sup>R</sup> clones was used as the template for PCR. A representative set of PCR results from six individual G418<sup>R</sup> clones is shown. Lane M, 1-kb molecular mass size ladder (GIBCO); lane -T, minus template control; lane U, negative control genomic DNA from uninfected HeLa cells. The correct 486-bp PCR product (arrow), diagnostic of loss of the intron, was detected in each clone (lanes 1A–100B; numbers denote MOI, letters denote individual clones). Lane V, 1361-bp unspliced product from HDAd-L1.3mneoI.

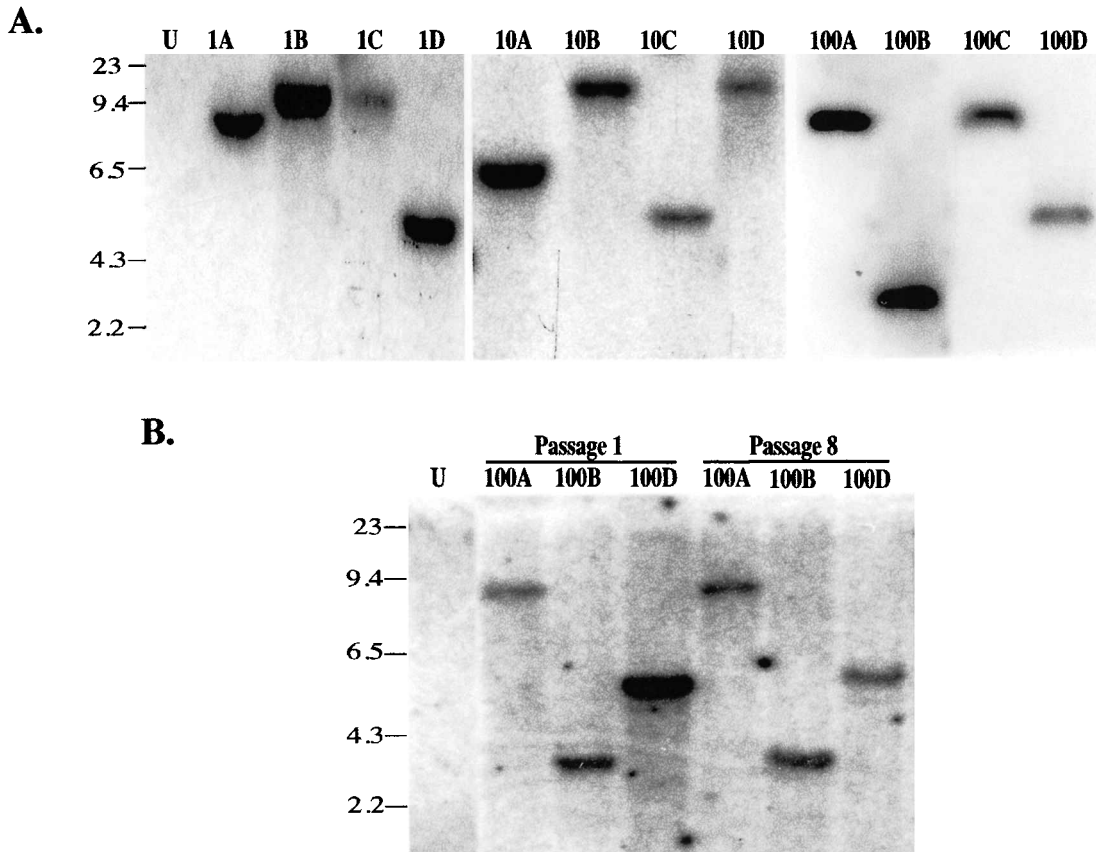
dicating that integrated transgene is stable (Fig. 3B). Moreover, no new hybridization signals were detected in the passage 8 clones, suggesting that additional retrotransposition events had not occurred even with prolonged serial passage after initial integration of the L1.3mneoI element.

#### *Quantification of integrating titers from the retrotransposon stage of the hybrid vector*

To accurately quantify the efficiency of retrotransposition after adenovirus-mediated delivery of the L1.3mneoI element, we first sought to correlate the functional measure of adenovirus transducing units at various MOIs with the physical copy number of introduced adenoviral genomes. Southern blots of total

genomic DNA from HeLa cells freshly infected at MOIs of 1, 10, and 100 were probed for the presence of the HDAd-L1.3mneoI helper-dependent adenovirus genome, and the intensity of the signal at each MOI was compared with a standard curve of increasing physical copy numbers (Fig. 4A). PhosphorImaging demonstrated a good correlation between functional MOIs and physical copy number, with infection at an MOI of 1 resulting in approximately 0.8 copy of HDAd-L1.3mneoI per cell, an MOI of 10 resulting in approximately 6 copies per cell, and an MOI of 100 resulting in approximately 65 copies per cell. Thus, increasing the MOI results in the delivery of correspondingly more copies of the retroelement to each adenovirus-infected cell.

We next determined whether delivery of increasing numbers



**FIG. 3.** (A) Transduction of HeLa cells with HDAd-L1.3mneoI results in stable integration of the *neo*<sup>R</sup> transgene. Approximately 20 mg of genomic DNA from 12 individual clones (MOI of 1, lanes 1A–1D; MOI of 10, lanes 10A–10D; MOI of 100, lanes 100A–100D) was digested with *Eco*RI and analyzed by Southern blot, using the *neo*<sup>R</sup> probe (Fig. 2A). Only one major restriction fragment of varying size is observed in each clone, indicating single retrotransposition/integration events had occurred. Lane U, genomic DNA from untransduced negative control HeLa cells.  $\lambda$ /*Hind*III ladder molecular weight values are depicted at left. (B) Integrated L1.3mneoI elements are stable. Three stable G418<sup>R</sup> clones were passaged an additional seven times under G418 selection (passage 1 and passage 8). Genomic DNA was collected at passage 1 and passage 8, digested with *Eco*RI and analyzed by Southern blot, using a *neo*<sup>R</sup> probe (Fig. 2A). The same size fragment hybridized to the probe in both passage 1 and passage 8 genomic DNA samples and no additional hybridization signals were detected in passage 8 genomic DNA.  $\lambda$ /*Hind*III ladder molecular weight values are depicted at left.

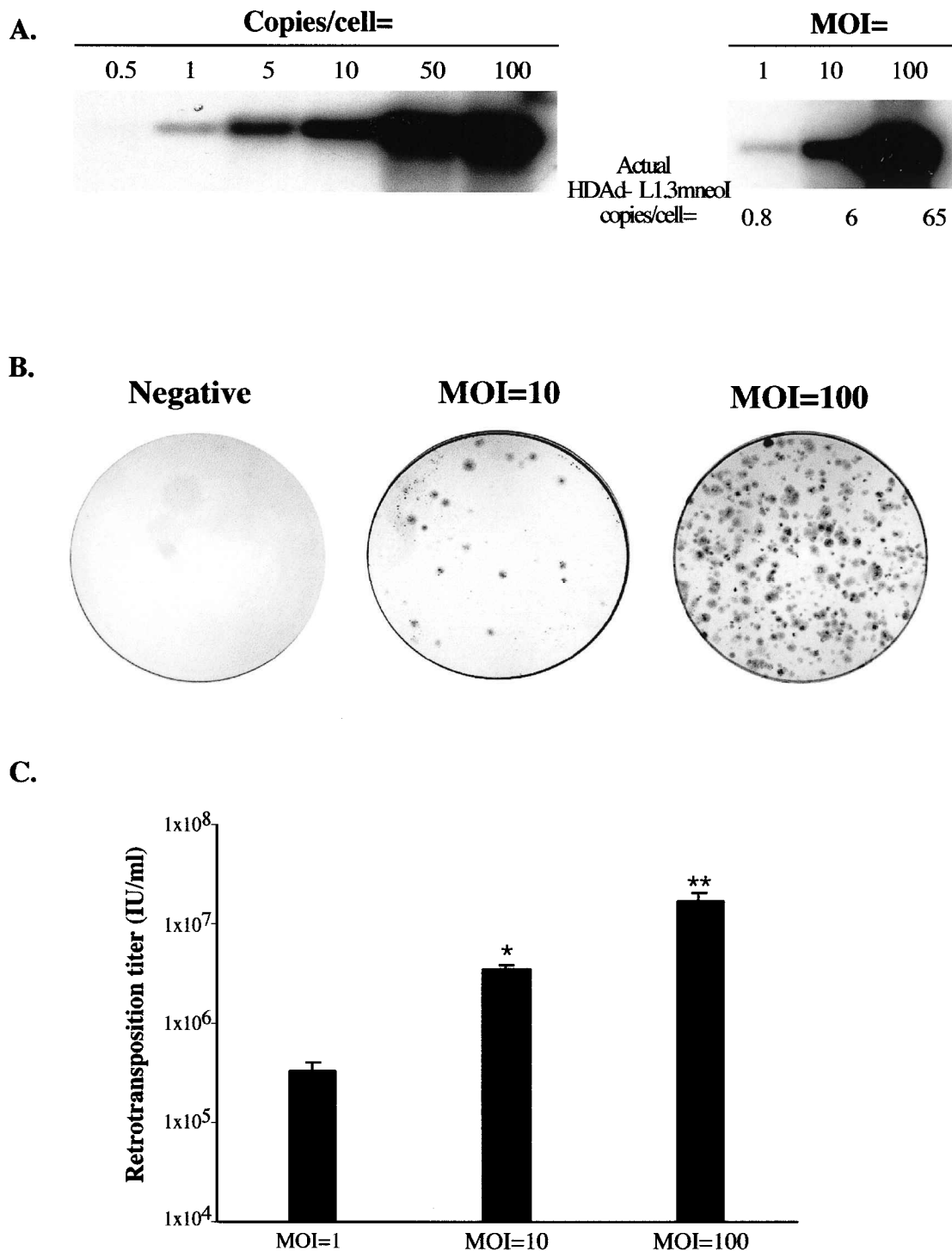
of the helper-dependent adenovirus stage of the hybrid vector results in higher frequencies of retrotransposition and transgene integration. HeLa cells infected with increasing MOIs of HDAd-L1.3mneoI were analyzed by fluorescence-activated cell sorting (FACS) 48 hr posttransduction to determine the number of adenovirus-transduced GFP-positive cells, and placed in G418 selection for 14 days. Once selection was complete, the number of G418-resistant colonies was counted to determine the frequency of retrotransposon integration events. The results of a representative experiment are shown in Fig. 4B, and demonstrate that the second-stage retrotransposon component of the hybrid vector is functional in conferring stable G418 resistance to infected cells. Furthermore, delivering more copies of the L1.3mneoI retroelement per cell by increasing the input MOI results in an increased retrotransposition frequency.

Using as a denominator the number of cells initially transduced by the first-stage HDAd-L1.3mneoI hybrid vector (determined by flow cytometric analysis of GFP expression), the frequency of the G418<sup>R</sup> phenotype was 1 in 14,286 cells con-

taining 1 copy of HDAd-L1.3mneoI. With 10-fold increases in virion copy number, the retrotransposition frequency increased to 1 event per 1382 transduced cells at 10 copies, and 1 event per 283 cells at 100 copies of HDAd-L1.3mneoI (Table 1). On the basis of the initial input titer of  $4.74 \times 10^9$  transducing units (TU)/ml for the first-stage HDAd-L1.3mneoI adenovirus, the titer of retrotransposition/integration of the second-stage L1.3mneoI element therefore corresponds to  $3.33 \times 10^5$  integrating units (IU) at 1 copy of HDAdL1.3neoI per cell,  $3.42 \times 10^6$  IU at 10 copies per cell, and  $1.68 \times 10^7$  IU at 100 copies per cell (Fig. 4C and Table 1).

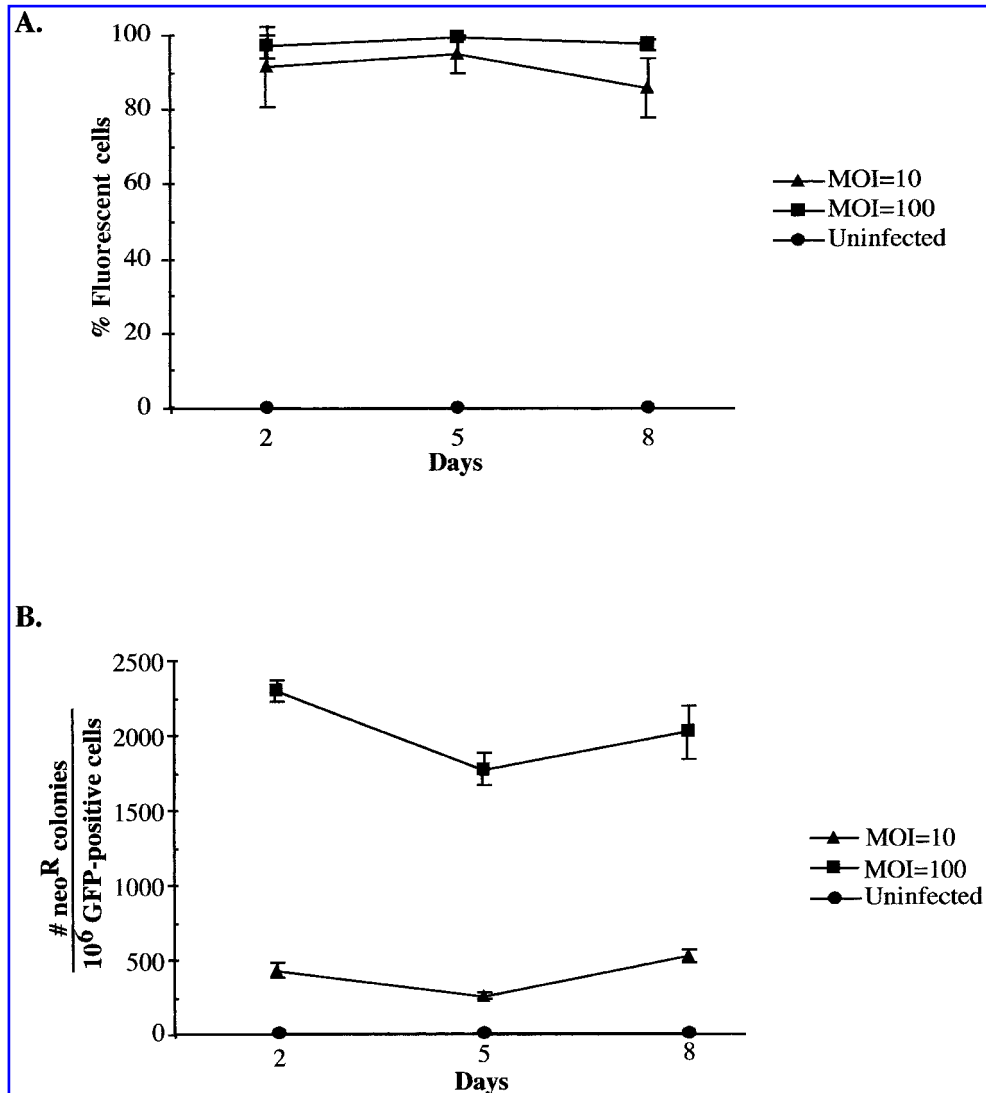
#### *Time course of retrotransposition events after helper-dependent adenovirus-mediated delivery of L1.3mneoI*

To determine whether L1 retrotransposition continues to occur as long as the first-stage adenovirus episome persists in the infected cells, we examined the time course of retrotransposition events after initial transduction. To prolong expression



**FIG. 4.** (A) Transduction with increasing MOIs correlates with delivery of increasing copy numbers of the HDAd-L1.3mneoI hybrid vector. Total genomic DNA was harvested from HeLa cells 24 hr posttransduction with increasing MOIs of HDAd-L1.3mneoI, digested with *ScaI*, and subjected to Southern blot analysis with a <sup>32</sup>P-labeled GFP fragment (*right*). The actual copy numbers of HDAd-L1.3mneoI delivered per cell at each MOI was determined by PhosphorImager analysis and comparison against a standard curve. The standard curve was generated by addition of plasmid DNA corresponding to the indicated copy numbers per cell to uninfected genomic DNA (*left*). (B) G418-resistant colonies generated from a representative retrotransposition assay. HeLa cells were infected with serial dilutions of HDAd-L1.3mneoI (MOIs of 10 and 100) and placed in G418-selection 48 hr posttransduction. Uninfected cells were selected in parallel as the negative control. G418 selection continued for 14 days, at which point the resistant colonies were fixed and stained with 0.4% Giemsa for visualization. (C) Higher copy numbers of HDAd-L1.3mneoI result in more efficient retrotransposition. The retrotransposition titer for each MOI was determined on the basis of the initial input adenovirus titer ( $4.74 \times 10^9$  TU/ml) and the retrotransposition frequency observed at each MOI, adjusted for the normalized copy number. \* $p < 0.05$ ; \*\* $p < 0.01$ .





**FIG. 5.** (A) Transduction at high MOIs results in prolonged expression from the HDAd-L1.3mneoI adenovirus episome. HeLa cells, transduced at MOIs of 10 and 100, were serially passaged and analyzed by flow cytometry for GFP expression on days 2, 5, and 8. More than 80% of cells continued to express GFP on day 8. (B) Retrotransposition frequency does not increase over time despite continued expression from the HDAd-L1.3mneoI episome. Subsets of HeLa cells were placed into G418 selection on days 2, 5, and 8 after transduction of the initial population at MOIs of 10 and 100. G418 selection was continued for 14 days, at which point the resistant colonies were fixed and stained with 0.4% Giemsa as described above. The number of G418<sup>R</sup> colonies obtained was normalized to 10<sup>6</sup> GFP-positive HeLa cells.

from the hybrid vector, we initially infected HeLa cells at MOIs of 10 and 100. At these higher MOIs, GFP expression from the first-stage adenovirus backbone persisted over a period of more than 8 days (Fig. 5A) in the absence of G418 selection.

We then determined L1 retrotransposition frequencies at various time points after initial transduction by HDAd-L1.3mneoI at MOIs of 10 and 100. The earliest time point assayed was on day 2 posttransduction, at which point the cells were counted and a defined number of transduced cells were placed in G418 selection for 14 days. This assay therefore measures the cumulative retrotransposition events occurring over a period of approximately 10 days, including the 8-day lag period before initiation of G418-mediated cell death plus the 2 days between transduction and the initiation of G418 selection. The remaining cells

were maintained without selection until the next time point. In the absence of G418 selection, the frequency of cells harboring early retrotransposition events should be maintained at a constant level relative to cells in which no retrotransposition has yet occurred (assuming both cell populations continue to divide at the same rate), unless the continued presence of HDAd-L1.3mneoI results in the accumulation of more cells that have newly integrated the *neo<sup>R</sup>* transgene. Therefore, additional aliquots from the originally transduced population maintained without selection, consisting of the same number of transduced cells each time, were similarly placed in G418 on day 5 and day 8 posttransduction and their retrotransposition frequencies were compared.

Interestingly, no increase in the retrotransposition frequency was noted with this assay when G418 selection was initiated

on days 2, 5, and 8 after the initial adenovirus infection (Fig. 5B). Thus, significant levels of additional retrotransposition do not appear to occur despite prolonged expression from the hybrid vector over the time course examined. This suggests that the majority of retrotransposition from the hybrid vector is a relatively early event occurring within the first 10 days after transduction.

## DISCUSSION

To effect the stable integration or episomal persistence of transgenes delivered via adenovirus vectors, a number of groups have reported the development of hybrid vector systems that contain retrovirus, adeno-associated virus (AAV), or Epstein-Barr virus (EBV)-derived elements within the context of standard E1-deleted or gutted adenovirus vectors (Fisher *et al.*, 1996; Bilbao *et al.*, 1997; Yoshida *et al.*, 1997; Caplen *et al.*, 1999; Recchia *et al.*, 1999; Tan *et al.*, 1999; Leblais *et al.*, 2000; Ueno *et al.*, 2000). In the present paper we describe the development of a unique retrotransposon-adenovirus chimeric vector system. Although use of retrotransposons as gene delivery vehicles has been previously suggested (Kingsman *et al.*, 1995; Hodgson *et al.*, 1997), and in fact retrotransposons such as rat VL30 elements have been found capable of being packaged and transmitted by murine leukemia virus (MLV) (Chakraborty *et al.*, 1994; Torrent *et al.*, 1994), the efficiency of delivering retrotransposon-encoded sequences to target cells has been the rate-limiting step. Moreover, while adenovirus-mediated delivery of a horn fly DNA transposase has been reported (Zhang *et al.*, 1998), actual transposition events after adenovirus infection of target cells were not demonstrated. Here we report the development of a novel hybrid vector that could achieve efficient delivery of a human retrotransposon in the context of a helper-dependent adenovirus, ultimately resulting in stable integration of a functional neomycin resistance transgene cassette. We further found that increasing virion copy number resulted in increased retrotransposition/integration titers that are comparable to the stable transduction titers achieved by other integrating vectors such as retroviruses.

In the present study we employed a retrotransposon vector derived from a human L1 element. These non-long terminal repeat (LTR) elements are among the most well-characterized human retrotransposons, and are present in approximately >500,000 copies in the human genome, although more than 99% of these are functionally inactive because of truncations and rearrangements, and of the remaining 3000 or so full-length L1 elements (Kazazian and Moran, 1998), it has been estimated that only about 1.5–2.5% (i.e., 30 to 60 copies) are active in retrotransposition (Sassaman *et al.*, 1997).

The retrotransposition frequency we observed from the HDAd-L1.3mneoI hybrid vector is in good concordance with that previously reported for this element from plasmid transfection studies. The specific L1 clone used in these studies, L1.3 (Dombroski *et al.*, 1993), has previously been reported to retrotranspose at a frequency of about 1 retrotransposition event scored per 150 cells (Sassaman *et al.*, 1997) when transfected into cells in the context of a self-replicating EBNA/*oriP* plasmid system (Moran *et al.*, 1996, 1999), which is maintained as an episome at 10 to 100 copies per cell (Yates *et al.*, 1985). In addition, our observed retrotransposition frequency is consis-

tent with that reported for L1.3 linked to a GFP transgene (Ostertag *et al.*, 2000), suggesting that the retrotransposition frequency is an intrinsic characteristic of the L1 element and not biased for G418 selection. With increasing MOI, we observed a linearly correlated increase in the frequency of retrotransposition; at 100 copies of HDAd-L1.3mneoI per cell, the retrotransposition frequency of L1.3mneoI was 1 retrotransposition event per 283 adenovirus-infected cells. Furthermore, this study represents the first use of an adenovirus-based hybrid vector system to achieve titratable delivery of a retrotransposon, allowing us to determine the inherent retrotransposition frequency for a single copy of the L1.3mneoI element as approximately 1 in 14,000 cells.

On the basis of the first-stage HDAd-L1.3mneoI adenovirus input titer of  $4.74 \times 10^9$  TU/ml, the second-stage L1.3mneoI retrotransposition/integration titer was therefore calculated to be on the order of  $3.33 \times 10^5$  IU at a physical copy number of 1 hybrid vector per cell. At higher MOIs, the integrating titer reached as high as  $1.68 \times 10^7$  IU. Thus, even in its present form the stable transduction efficiency of this hybrid vector still compares favorably with those of other integrating vectors such as retroviruses. Furthermore, a new L1 element, L1<sub>RP</sub>, has been discovered (Kimberland *et al.*, 1999), which shows the highest retrotransposition frequency (up to 1 event per 30 cells) observed to date. Employing such elements in future generations of retrotransposon-adenovirus hybrids will enhance the efficiency of this vector system.

Only single integration events were observed in the majority of subclones examined, even when HDAd-L1.3mneoI infections were performed at a high MOI. This suggests that multiple retrotransposition events do not occur frequently even when multiple copies of the L1.3mneoI element are introduced into the target cells, using a nonreplicating vector. The low abundance of clones with multiple integration events (1:20 clones) is in contrast to another report, which demonstrated that HeLa cells can accommodate multiple L1 insertions after plasmid transfection (Wei *et al.*, 2000). However, this latter result may be due to a quantitative difference in the number of retrotransposon copies transiently introduced into target cells at the time of chemical transfection, which would be far greater than the approximately 65 copies introduced by HDAd-L1.3mneoI even at an MOI of 100.

That the initial copy number is of primary importance in determining the overall retrotransposition frequency is also supported by our observation that the majority of retrotransposition appears to occur within a relatively early time period after hybrid vector-mediated introduction of the L1 element. Although we did not detect an increasing retrotransposition rate with longer expression of the retroelement after HDAd-L1.3mneoI-mediated transduction, it has been demonstrated that the L1.3 element can continue to retrotranspose at a rate of about 0.1% of transfected cells per day when expressed from a self-replicating plasmid (Ostertag *et al.*, 2000) in hygromycin-selected cells. In the latter study, the GFP-based indicator cassette employed in the transfection studies might make possible the more sensitive detection of additional retrotransposition in stably selected cells; moreover, there was no selection for the HDAd-L1.3mneoI adenovirus in our experiments.

We further observed that, while HDAd-L1.3mneoI-mediated transduction was capable of giving rise to G418 resistance, additional retrotransposition events did not occur from L1.3mneoI elements that had already integrated, even on prolonged serial

passage of the transduced cells. This result is consistent with previous analyses of integrated L1/reporter elements, which demonstrated a high frequency of truncated ORF 1 and ORF 2 sequences at their 5' ends, possibly because of the low processivity of the L1-encoded reverse transcriptase (RT) (Moran *et al.*, 1996; Kazazian and Moran, 1998). The propensity toward truncations at the 5' end of L1 element is actually an advantage for an L1-hybrid vector for gene delivery because it provides a built-in safety mechanism that greatly diminishes the possibility of additional rounds of retrotransposition occurring from previously integrated L1/transgene elements. Furthermore, one study demonstrated that heterologous L1 and cellular RNAs are mobilized at much lower frequencies (<1%) when compared with tagged, wild-type L1 clones, using a transfection system. Thus, in contrast to mariner-like DNA transposases, L1-encoded proteins demonstrate a profound *cis* preference for their encoding RNA (Wei *et al.*, 2001), so that expression of L1 ORF products in human cells per se will not lead to rampant *trans*-activation of endogenous L1 elements. This *cis* preference further suggests that L1-based gene delivery systems will be relatively safe, and there is likely to be little chance of promiscuous recurrent L1 retrotransposition leading to unacceptable frequencies of insertional mutagenesis.

One distinct advantage of this retrotransposon-adenovirus hybrid vector system is that the same cell that was originally transduced by the first-stage adenovirus vector will itself be permanently transduced by the second-stage L1 retrotransposon vector. In contrast, retrovirus-adenovirus hybrid vector systems that have been previously described require the initially transduced cell to serve as a packaging intermediate for generation of the second-stage retroviruses, which must then go forth to permanently transduce adjacent cells (Bilbao *et al.*, 1997; Caplen *et al.*, 1999). Another potential advantage of this retrotransposon-adenovirus hybrid vector system is that L1 elements are normally present endogenously in all human cells, making a cellular immune response directed against the second-stage L1 retrotransposon components less likely to occur. In addition, as the helper-dependent adenovirus vector used to deliver the L1 element is itself deleted of all the adenoviral genes, the first-stage adenovirus carrier will be less toxic as well (Morral *et al.*, 1998, 1999). Therefore this type of retrotransposon-adenovirus hybrid vector may provide a high-titer adenovirus-based system that is potentially less immunogenic, yet capable of mediating stable integration and permanent transduction of target cells.

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