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Integration efficiency of a hybrid adenoretroviral vector

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Abstract

The frequency with which the hybrid vector AdLTR-luc mediates genomic integration [Nat. Biotech. 18 (2000) 176–180] is unknown. To address this, we constructed AdLTR-red, using the AdLTR-luc backbone and the enhanced red fluorescence protein cDNA. Kinetic studies showed that AdLTR-red and a conventional adenoviral vector, AdCMV-red, entered and transduced epithelial cells comparably. AdLTR-red integration frequency in vitro, i.e., the percentage of red clones after 10–12 doubling times from limiting dilutions, was 8.0% (36/450; at 67 particles/cell). With AdCMV-red, 0/549 clones were integration-positive. Seven days after AdLTR-luc or AdCMV-luc (10^{11} particles) femoral vein administration to adult rats splenocytes were prepared, stimulated with concanavalin A, and examined by FISH. AdLTR-luc integration occurred in 5–11% of mitotic rat splenocytes, while no unequivocal integration was found with AdCMV-luc. These data provide the first quantitative evidence of the frequency for genomic integration with this hybrid vector.
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Keywords: Hybrid vector; Adenovirus; Retrovirus; Integration; Non-homologous recombination

Previously, we reported the construction of a prototype hybrid adenoretroviral vector, AdLTR-luc [1], capable of infecting a wide variety of dividing and nondividing cell types, yet able to mediate integration of the transgene cassette into genomic DNA. However, the frequency of this integration event was not rigorously quantified. AdLTR-luc should present to a target cell as a conventional adenoviral vector. Adenoviruses are lytic viruses and integrate into a host cell genome at very low frequency (10^{-3}–10^{-6}) after infecting mammalian cells in vitro [2,3]. AdLTR-luc is based on a first generation $E1^-$ adenoviral backbone, with *cis*-acting elements from Moloney murine leukemia virus (MoMLV) in a unique arrangement flanking the transgene cassette (see Fig. 1 for general structure). Upstream of the cassette is a (5′–3′) sequence containing part of the envelope gene (1.5 kb), the entire 5′ long terminal repeat (LTR, 0.57 kb), and the packaging sequence (0.63 kb). Downstream of the cassette is a small portion of the envelope sequence (~0.5 kb) and the intact 3′LTR (0.57 kb). The present study was designed to provide critical information [4] on the frequency of genomic integration for this hybrid vector in comparison to a conventional adenoviral vector.

Materials and methods

Recombinant viral vectors. AdLTR-luc and AdCMV-luc have been described earlier [1,5]. AdLTR-red (Fig. 1A) possesses a similar backbone to AdLTR-luc, however, the enhanced red fluorescence protein (ERFP) cDNA (from pDsRed1-N1, Clontech Laboratories, Palo Alto, CA) is used as the reporter gene. AdCMV-red (Fig. 1B) is a conventional first generation adenoviral vector containing the CMV promoter and the ERFP cDNA, and is similar to AdCMV-luc. AdLTR-red and AdCMV-red were generated by homologous recombination [6] of pACLTR-red and pACCMV-red with the pJM17 plasmid in C7 cells (a gift of Dr. J. Chamberlain, University of Michigan) [7,8].

Quantitative PCR. Particle titers were determined using quantitative PCR (Q-PCR) assays, carried out with SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA), the ABI Prism 7700 Sequence Detector (Applied Biosystems) and adenoviral E2 gene primers. The primers E2q1 (5′-GCAGAACCACCAGCACAGTGT-3′) and E2q2 (5′-TCCACGCATTTCCTTCTAAGCTA-3′) were used to amplify 72 bp of the adenoviral E2 gene (from 4483 to 4554 bp of Ad5). The titers for the vector preparations used herein were as follows: AdLTR-luc (6.7×10^{13} particles/ml), AdCMV-luc (5.8×10^{13} particles/ml),

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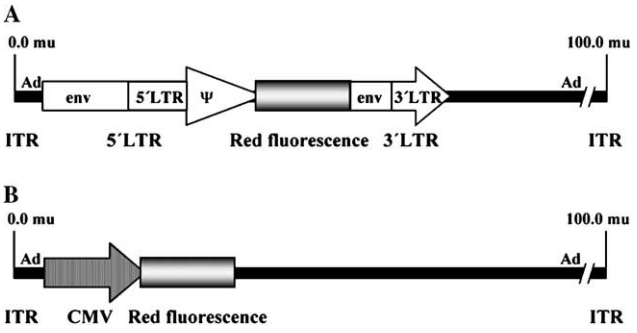

Fig. 1. Structure of recombinant adenoviruses used. (A) AdLTR-red. Upstream of the transgene, AdLTR-red contains 2.7 kb MoMLV sequence, including part of the envelope gene [1.5 kb], the 5′LTR [0.57 kb], and the packaging sequence [0.63 kb]. Downstream of the transgene, AdLTR-luc contains 1 kb MoMLV sequence, including a small part [~0.5 kb] of the envelope gene and an intact 3′LTR. (B) AdCMV-red is a conventional, first generation adenoviral vector.

AdLTR-red (5.8×10^{11} particles/ml), and AdCMV-red (8.9×10^{11} particles/ml). A titer based on red particles was measured by Q-PCR using the primers redq3 (5′-ATCAAGGAGTTCATGCGCTTC-3′) and redq4 (5′-CTCGAACTCGTGGCCGTT-3′) to amplify 63 bp of the ERFP cDNA.

Cell culture. The A5 epithelial cell line was grown as described [9]. A5 cells do not transcomplement $E1^-$ adenoviruses [5]. The 293 cell line contains an integrated copy of the left-most 62% of the adenovirus 5 genome and complements the deficiency in $E1^-$ vectors [10]. C7 cells are derived from 293 cells but also stably express the adenovirus 5 preterminal protein and DNA polymerase [7,8].

In vitro experiments. For the time course of AdLTR-red and AdCMV-red entry into cells, 100–200 E2-based particles were delivered per 293 cell for 0, 15, 30, 60, 90, or 120 min. To examine the concentration dependence of AdLTR-red and AdCMV-red entry into 293 cells, different vector concentrations (see Fig. 2) were used to infect 293 cells for 120 min. After the indicated time of infection, cells were immediately washed three times with phosphate-buffered saline (PBS) and genomic DNA was extracted with the Wizard Genomic DNA extraction kit (Promega, Madison, WI) for use in Q-PCR assays.

To determine the transduction efficiency of vectors, A5 cells were infected in suspension at 37 °C for 1 h (see Fig. 3 for doses). After infection, 8×10^6 cells were plated into one well of a 2-well permanox slide (Nalge Nunc International, Naperville, IL) and cultured for 3 days. The cell number for plating was chosen to allow the rapid development of a confluent monolayer and minimize cell proliferation. On day 3, cells were harvested with trypsin/versene (Biosource International, Rockville, MD) and washed three times with PBS, and flow cytometry was performed using a FACS Calibur equipped with Cell Quest software (Becton–Dickinson, San Jose, CA) to quantify the proportion of cells expressing ERFP.

Animal experiments. Animal experiments were approved by the NIDCR Animal Care and Use Committee and conducted in accordance with IASP standards for the treatment of rats. Rats were infected with vectors by femoral vein (10^{11} particles) and seven days later whole spleens (three rat spleens for each virus group, AdLTR-luc or AdCMV-luc) were pooled, placed into cold PBS, and sent for FISH assays. FISH assays were carried out by SeeDNA Biotech (Windsor, Ontario, Canada) in a blinded manner [1,11]. The luciferase cDNA (2.7 kb) was used as a probe. This probe was biotinylated with dATP using the BRL BioNick labeling kit. FISH experiments with AdLTR-luc infected spleens were repeated 3 times, while those with AdCMV-luc infected spleens were repeated twice (see Table 2).

To estimate the number of viral particles present in spleen samples seven days post-infection, genomic DNA was extracted from infected rats and Q-PCR assays were carried out as above. The primers used

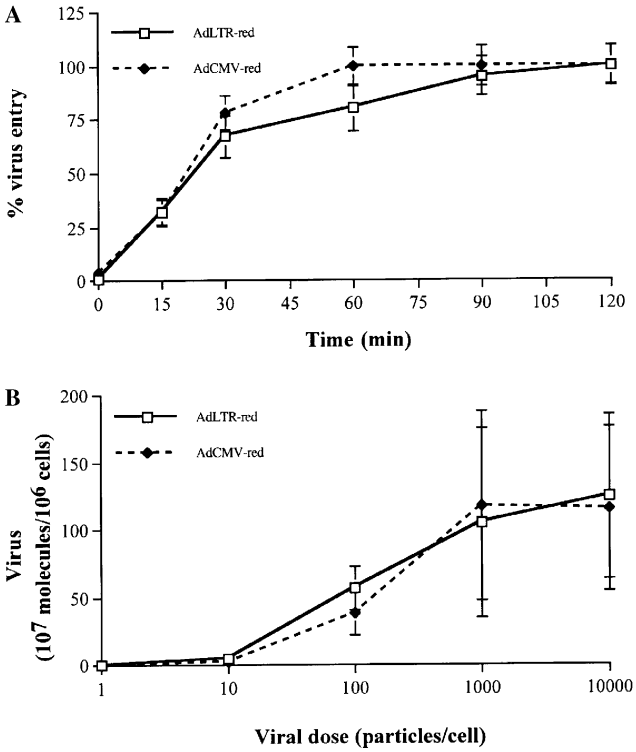

Fig. 2. Viral entry into 293 cells. (A) Time course of AdLTR-red and AdCMV-red entry into cells. One hundred–two hundred E2-based particles were delivered per 293 cell for the indicated times. Data are shown as the percentage of added virus entering the cells. Results are the means ± SEM for three separate experiments, each performed in triplicate. (B) Concentration dependence of AdLTR-red and AdCMV-red entry into 293 cells. Different vector concentrations were used to infect 293 cells for 120 min. Data represent the means ± SEM for three separate experiments, each performed in triplicate. After the indicated time of infection, genomic DNA was extracted and used in Q-PCR assays.

Table 1
Determination of integration efficiency in cell clones in vitro

Virus infection (red particles/cell)	AdLTR-red			AdCMV-red		
	Total clones	Red clones	% Red clones	Total clones	Red clones	% Red clones
27	675	9	1.3	224	0	0
67	450	36	8.0	549	0	0

A5 cells were infected with AdLTR-red or AdCMV-red, as indicated, in suspension at 37 °C for 1 h, and then distributed onto 96-well plates at ~0.5 cells/well. Thereafter, cells were followed by visual inspection with a fluorescence microscope for 8–10 days (10–12 doubling times). Data are the average of two separate experiments.

were lucqlF (5′-GGCGCGTTATTTATCGGAGTT-3′) and lucq2R (5′-TACTGTTGAGCAATTCACGTTCATTAT-3′) to amplify 72 bp of the luciferase cDNA. There were on average 10^9, and 1.1×10^9, viral particles/spleen after infection with AdCMV-luc and AdLTR-luc, respectively (i.e., ~1% of all viral particles administered were present in the spleen on day 7). Spleens were homogenized and Ficoll-Paque was used for spleen cell separation. On average spleens contained $\sim 1.4 \times 10^8$ cells, with $\sim 2 \times 10^7$ mononuclear cells theoretically available for FISH analyses.

Luciferase assay. About 50 μg rat liver was homogenized in 500 μl cell lysis buffer (Promega) for 15 s and left to stand at room temperature for 15 min. Cell lysates (50 μl) were then added to 100 μl of luciferase substrate and light output (relative light units, RLU) was measured in a luminometer.

Southern blot hybridization. Three months following vector infection, cloned A5 cells (five clones each with either AdLTR-red or AdCMV-red; from experiments shown in Table 1) were used to obtain genomic DNA with a Non-organic DNA Extraction kit (Intergen, Purchase, NY). Generally, 15 μg of genomic DNA from each sample was digested with the indicated restriction enzymes and separated on a 1% agarose gel. Nucleic acids were then transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). These blots were hybridized with a ^{32}P-radiolabelled ERFP cDNA probe (a 423 bp *Nco*I fragment) and autoradiographed.

Results

In vitro experiments

We first examined the kinetics of viral entry into 293 cells using AdLTR-red and AdCMV-red. As shown in Fig. 2A, the time course exhibited by both vectors was similar. In general, the rate of vector entry tended to diminish after 30–60 min. We next examined the vector concentration dependence of this entry process, exposing 293 cells to AdLTR-red or AdCMV-red over a concentration range from 1 to 10,000 particles/cell for 120 min. As shown in Fig. 2B, vector entry was concentration dependent, and quite similar for both the hybrid and conventional vectors. The EC_{50} was ~500 particles/cell and a maximal value was achieved between 1000 and 10,000 particles/cell. Although not unexpected, these two types of experiments clearly show for the first time that the ability of hybrid and conventional vectors to enter 293 cells is quite comparable.

To determine the transduction efficiency of each vector in vitro, A5 epithelial cells were infected with AdLTR-red and AdCMV-red at different doses for 3 days. Thereafter, cells were examined by flow cytometry to determine the proportion of red cells present at each vector dose. As shown in Fig. 3A, both AdLTR-red and AdCMV-red transduction of A5 cells was quite similar. The EC_{50} was achieved at a concentration of ~100–200 vector particles/cell, while both vectors achieved maximal transduction at $\geqslant$500 particles/cell. Under our experimental conditions, 82.4% of cells were red at maximum after AdLTR-red infection, while 85% of cells were red at maximum after AdCMV-red infection. As is clear from the distribution pattern of transduced cells

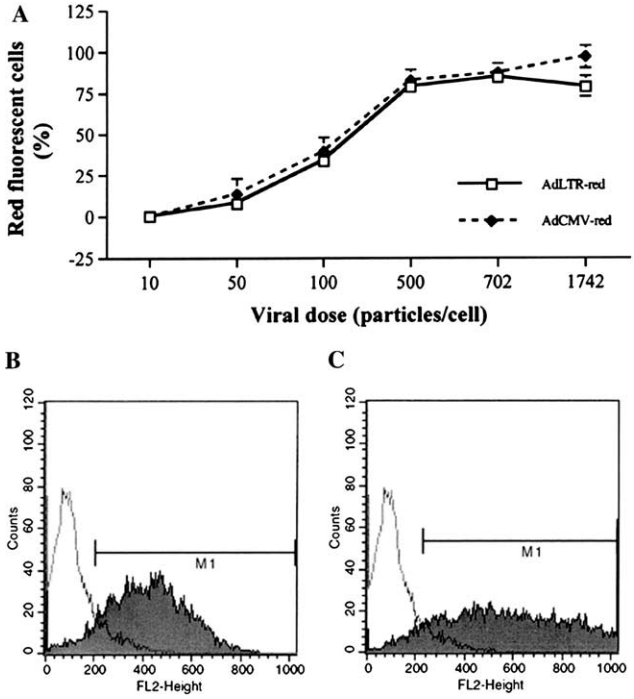

Fig. 3. Determination of transduction efficiency of vectors in A5 epithelial cells. (A) Vector concentration dependence (based on E2 particles) of cellular transduction. Data represent the percentage of red cells transduced with either AdLTR-red or AdCMV-red (mean values ± SEM) for two separate experiments, each performed in triplicate. (B and C) Representative results of FACS analyses of cells transduced with either AdLTR-red (B) or AdCMV-red (C) at 500 E2 particles/cell. Solid line represents uninfected cells. Shaded area represents the red cell population, equivalent in B to 82.4% of cells and equivalent in C to 85% of cells.

shown in Figs. 3B and C, infection of cells with AdCMV-red led to more cells expressing higher levels of ERFP. These results are not surprising since the CMV promoter is a much stronger promoter than the MoMLV LTR in an adenoviral context [1,12]. These experiments clearly demonstrate that the transduction efficiency of AdLTR-red is quite similar to that of a conventional adenoviral vector, i.e., unaffected by inclusion of the indicated retroviral elements, as was theoretically expected.

We next infected A5 cells with AdLTR-red and AdCMV-red, at either 27 or 67 red genome-containing particles/cell, to determine the frequency of integration. In addition to being unable to transcomplement $E1^-$ adenoviral vectors, A5 cells double rapidly, ~ every 18 h [5]. One hour after infection, cells were washed, then plated into 96-well plates at ~0.5 cell/well, and cultured for 8–10 days (10–12 doubling times). Thereafter, we measured the number of ERFP-positive clones with fluorescence microscopy (Table 1). We found that 9 out of 675 (1.3%) clones isolated after infection with AdLTR-red at 27 particles/cell were ERFP-positive. When cells were infected with this vector at 67 particles/cell, 36 out of 450 isolated clones (8.0%) were ERFP-positive.

Conversely, none of the clones obtained after infection with AdCMV-red (224 examined at 27 particles/cell and 549 examined at 67 particles/cell) exhibited red fluorescence (Table 1). Several of the clones derived from AdLTR-red infected cells have now been grown in culture for ~8 months and have continued to exhibit comparable levels (>95% of cells) of red fluorescence.

To confirm that the above results reflected genomic integration of the ERFP cDNA, we selected five clones derived from AdLTR-red infected cells and five clones from AdCMV-red infected cells for further study. After amplification of cells, genomic DNA was extracted, digested by restriction endonucleases, electrophoresed, and then hybridized with a 423 bp ERFP probe. Following digestion with *Bam*HI, all five clones infected with AdLTR-red possessed the intact 964 bp ERFP cDNA and SV40 poly(A) sequence (Fig. 4). Conversely, none of the samples obtained from clones infected with AdCMV-red contained the ERFP cDNA. When samples from AdLTR-red infected clones were digested with either *Bgl*I, *Bgl*II or *Xho*I, all showed evidence of ERFP cDNA genomic integration, exhibiting hybridization-positive bands, different in size from those obtained with the positive control sample (Fig. 4). At least one AdLTR-red infected clone appeared to experience two integration events (#3).

In vivo experiments

We extended these studies to estimate the frequency with which the hybrid vector integrates into genomic DNA in vivo. For these experiments we used AdLTR-luc and AdCMV-luc as in our previous studies [1] and infected rats via a femoral vein injection. To demonstrate that rat tissues were successfully infected by injection of these vectors, we measured luciferase activity in livers obtained at the time of sacrifice (on day 7). In a typical experiment, the livers from rats whose spleen cells were to be examined by FISH showed on average ~4305 RLU/25 μg protein after AdLTR-luc administration, and ~10,130 RLU/25 μg protein after AdCMV-luc delivery ($n = 3$ rats/vector). For all spleen cells to be examined by FISH, the corresponding liver samples showed luciferase activity at seven days post-infection.

FISH assays were performed on splenocytes from rats on day 7 post-vector infection and assessed in a blinded manner (Table 2). For AdLTR-luc, 3 experiments were carried out between 1999 and 2001. For experiments 2 and 3, AdCMV-luc was tested concurrently with AdLTR-luc. In aggregate, 600 mitotic mononuclear cells were counted for the AdLTR-luc infected group. Of these, positive FISH signals were seen in 40 cells for an average frequency of integration in vivo

Table 2
Frequency of positive integration in spleen cells in vivo

Viruses	Experiment number		
	1 (%)	2 (%)	3 (%)
AdCMV-luc	ND	<1*	0
AdLTR-luc	5.0	5.7	11.0

The data are presented as the percentage of total mitotic rat spleen cells examined (100–200 cells/virus/experiment) that exhibited a positive FISH signal (allelic pair). See Materials and methods for further details. * Two "blinded" examiners evaluated the cells in this experiment and one scored a single cell as positive, while the other did not. ND, not done concurrently.

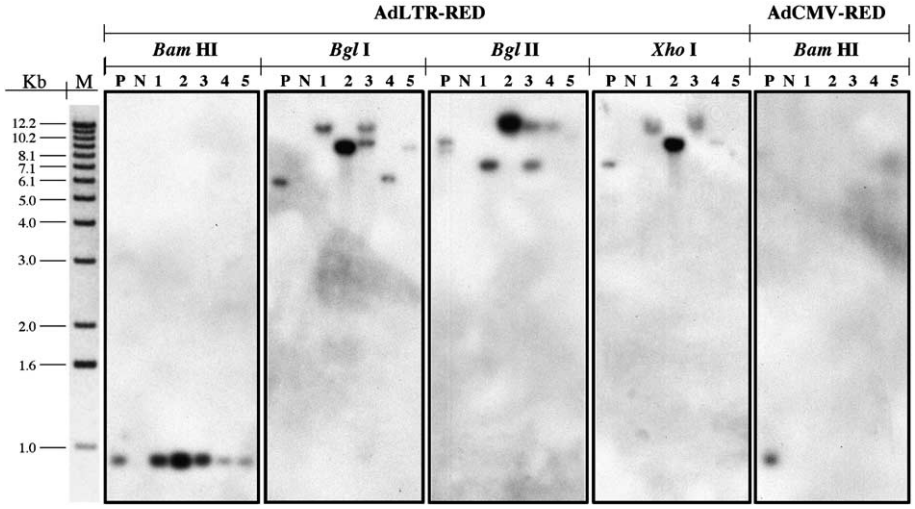

Fig. 4. Southern blot hybridization analyses for the integration of the enhanced red fluorescence protein cDNA. Cloned A5 cells (five obtained after infection with AdLTR-red and five obtained after infection with AdCMV-red), three months after vector infection (from experiments shown in Table 1), were used to extract genomic DNA. After digestion with the indicated restriction endonucleases, Southern blot analyses were performed as described in Materials and methods. The positive control (P) was AdLTR-red or AdCMV-red viral genomic DNA. The negative control (N) was genomic DNA extracted from uninfected A5 cells. The migration position of standard DNA fragments (M) is shown to the left.

of ~7%. A total of 250 mitotic mononuclear cells were counted for the AdCMV-luc group. Only one mitotic cell was identified as positive, and this was equivocal (see Table 2 legend), for a maximal possible integration rate of 0.4%.

Discussion

As lytic viruses, adenoviruses integrate only at very low frequency (10^{-3}–10^{-6}) in cultured mammalian cells [2,3]. Thus, it was not surprising that in our studies with the conventional adenoviral vectors AdCMV-red and AdCMV-luc we were unable to detect any unequivocal integration events. However, as noted earlier, when epithelial cells were infected in vitro with AdLTR-red, integration events were readily observed (Table 1, Fig. 4). Furthermore, when we studied AdLTR-luc integration in vivo, we observed consistent integration frequencies (between 5% and 11%) in three separate experiments with rat spleen cells carried out over ~2.5 years.

The mechanism by which AdLTR-luc or—red achieves genomic integration is not understood. These chimeric vectors include the *cis* elements of the retrovirus non-homologous recombination system (albeit in an unusual order) without the generally required *trans* factor, MoMLV integrase [1,13]. Integration by this prototype hybrid vector is integrase-independent and takes place randomly in the genome [1]. We have hypothesized that the unique arrangement of retroviral elements employed in these hybrid vectors interacts with host cell nuclear proteins to facilitate a novel, non-homologous genomic recombination [14]. Interestingly, we found specific gel mobility shifts when DNA fragments included the 3′ end of the *env* region and the 5′ half of the upstream LTR [14]. This segment also includes the previously reported 404 bp region within which integration breakpoints occur [1].

Consistent with our hypothesis, a search of human and rat genomic databases revealed no sequences corresponding to the MoMLV elements employed (Zheng, not shown). In addition, it is recognized that retroviral DNA elements can utilize numerous host cell proteins to promote provirus formation [15,16]. For example, HMGI (Y) appears to function as an architectural co-factor in this process [17], while several host cell DNA repair enzymes (e.g., poly[ADP-ribose] polymerase, polymerase beta, PCNA, flap endonuclease, ligases I, III or IV, and DNA-dependent protein kinase) also support retroviral DNA integration [15,16]. Furthermore, it is known that adenoviral E4 proteins can interact with, and inhibit, several host cell DNA repair proteins [18,19].

More study is needed to clarify how the *cis* acting retroviral sequences incorporated into these hybrid vectors are able to facilitate integration. However, the experiments presented herein clearly show that while these hybrid vectors infect cells similar to conventional adenoviral vectors, they integrate into the genome at frequencies much greater than those achieved by the conventional vectors.

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