Biochemical and Biophysical Research Communications **291,** 34–40 (2002)
doi:10.1006/bbrc.2002.6401, available online at http://www.idealibrary.com on IDEAL®

Long-Term Expression after Infection by the Hybrid Vector AdLTR-luc Is from Integrated Transgene

Changyu Zheng and Bruce J. Baum[1]

Gene Therapy and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892-1190

Received December 21, 2001

The novel adenoretroviral vector, AdLTR-luc, infects dividing and nondividing cells, and mediates long-term transgene expression(Zheng, C., Baum, B. J., Iadarola, M. J., and O'Connell, B. C., *Nat. Biotech.* 18, 176–180, 2000). To determine the source of this expression we examined two epithelial cell lines. One, HSG, permits E1⁻ recombinant adenoviral replication, while the other, A5, does not. An HSG clone, that expressed luciferase stably for >6 months, was obtained following infection at ~0.2 AdLTR-luc particles/cell. Southern and PCR analyses showed that luciferase cDNA present was integrated. A5 cells were infected with AdLTR-luc at ~1000 particles/cell, and colonies were obtained by limiting dilution. Eight clones showed stable luciferase activity for >9 months. High molecular weight DNA extracts from clones were positive for genomic integration by Southern, PCR, and quantitative PCR analyses. Similar analyses of low molecular weight DNA extracts indicated the absence of intact extrachromosomal vector. These data demonstrate that long-term luciferase expression after infection by AdLTR-luc is derived from the integrated cDNA.

Key Words: **gene therapy; integration; adenoretrovirus; hybrid vector; long-term gene expression; extrachromosomal DNA.**

Recently, we reported the construction of a prototype hybrid adenoretroviral vector, AdLTR-luc, capable of infecting a wide variety of dividing and nondividing cell types, like adenoviral vectors, yet able to mediate integration of the transgene cassette into genomic DNA, like retroviral vectors (1). This hybrid vector is based on a first-generation E1⁻ adenoviral backbone, with elements from Moloney murine leukemia virus (MoMLV) flanking the expression cassette (Fig. 1). To determine if this hybrid vector can be useful for gene therapy applications, several important questions remain to be answered (2): (i) Is the long-term transgene expression derived from integrated or extrachromosomal forms of the vector? (ii) What are the infection and integration efficiencies of AdLTR-luc? and (iii) What is the mechanism by which integration occurs? The present report addresses the first of these key questions.

Extrachromosomal DNA has potential to integrate into the cellular genome (3). Nonhomologous recombination is one pathway by which such integration proceeds. Different viruses integrate by nonhomologous recombination and, in order to improve the frequency of successful integration, have evolved specialized systems. These systems can be organized into *cis* and *trans* elements. The *trans*-acting factors are often virus-encoded proteins that catalyze DNA recombination, e.g., MoMLV or HIV integrase. The *cis*-acting components are DNA substrates that are part of the virus genome, e.g., MoMLV or HIV long terminal repeats (LTRs: 4, 5).

Adenovirus is a lytic virus that integrates at very low frequency (6). First generation adenoviral vectors primarily stay in an extrachromosomal location in the nucleus, and after adenoviral infection gene expression comes from extrachromosomal vectors almost entirely. MoMLV vectors integrate into the host cell genome, facilitated by *cis* and *trans* elements. MoMLV integration requires both LTRs along with a viral encoded integrase (7, 8). Gene expression is derived entirely from integrated provirus forms. AdLTR-luc includes some *cis* elements of MoMLV vectors. Recently (1), we showed that the frequency of integration with AdLTR-luc is significantly greater than that for first generation adenoviral vectors. The results herein show that long-term luciferase gene expression after AdLTR-luc infection derives from integrated transgene.

MATERIALS AND METHODS

Recombinant viral vectors. The replication-deficient recombinant adenoviral vectors used are based on the adenovirus type 5 genome.

[1] To whom to address correspondence and reprint requests should be addressed at GTTB, NIDCR, NIH, Building 10, Room 1N113, Bethesda, MD 20892-1190. Fax: (301) 402-1228. E-mail: bbaum@dir.nidcr.nih.gov.

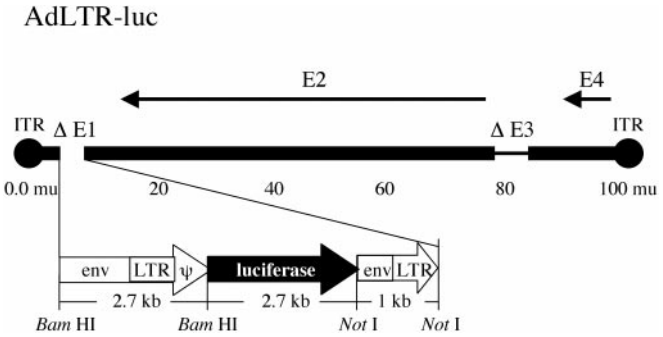


FIG. 1. Structure of AdLTR-luc. See Materials and Methods for details.

To construct AdLTR-luc, we added 2.7 kb of MoMLV sequence 5′ to the transgene (including, in a 5′ to 3′ direction, part of the envelope gene [1.5 kb], the 5′LTR [0.57 kb], and the packaging sequence [0.63 kb]) and 1 kb of MoMLV sequence 3′ to the transgene (including a small part, ~0.5 kb, of the envelope gene and an intact 3′LTR) (for details see Ref. 1). The MoMLV sequences were cleaved by *Eco*RI from the plasmid pXT1 (Stratagene, La Jolla, CA; 9). The 2692 bp luciferase open reading frame plus poly(A) sequence was cleaved from the plasmid pGL2-Basic (Promega, Madison, WI). AdLTR-luc (Fig. 1) was generated by homologous recombination of pACLTR-luc (1) with pBHG10 (Microbix Biosystems Inc., Toronto, Canada) in 293 cells (10). AdCMV-luc, a first generation $E1^-$ adenoviral vector, was constructed as described (11) and used here to check viral replication in A5 and HSG cells (see below). In contrast to AdLTR-luc, AdCMV-luc does not mediate genomic integration (1). Both viruses were propagated using 293 cells. Plaque assays to determine viral titers were carried out in 293 cells.

Cell culture, cellular infection, and isolation of cell clones. The A5 cell line (12) was derived from rat submandibular gland, and grown in McCoy's 5A medium. The HSG cell line was derived from an irradiated human submandibular gland (13; a gift from Professor M. Sato, Tokushima University, Japan), and grown in DMEM/F12 (1:1) medium. The 293 cell line is from human embryonic kidney (14) and complements the deficiency in $E1^-$ vectors.

To obtain infected, cloned cell lines, A5 cells were infected in suspension with AdLTR-luc at 1000 particles/cell, while HSG cells were infected with AdLTR-luc at 0.2 particle/cell. At 1 h postinfection, A5 cells were distributed onto 96-well plates at ~0.5 cell/well, and thereafter followed by visual inspection. After 14 days, cells in many wells were confluent, and subsequently all cells from a well were transferred to a single well of a 24-well plate. After confluence was attained in 24-well plates, 2% of the cell sample was used to check luciferase activity. Eight luciferase positive clones with relatively high luciferase activity (from 116 clones obtained) were selected for further long-term study. Only one luciferase positive HSG cell clone, out of 398 obtained, was identified. That clone has subsequently been kept in continuous cell culture.

Luciferase assay. Cells were homogenized and lysed in cell lysis buffer (Promega) for 15 min. Fifty microliters of the cell lysate was added to 100 μl of luciferase substrate, and light output was measured with a luminometer. Results are expressed as relative light unit (RLU) per cell number.

PCR assays. Genomic (high molecular weight) DNA used as a template in PCR assays was extracted with the Non-Organic DNA Extraction kit (Intergen, Purchase, NY). We used the method of Hirt (15) to extract low molecular weight DNA. One μg of template DNA was used in each PCR. The PCR product was 2445 bp of the luciferase cDNA, amplified by the primers lucF1 (5′-CGCTGGAGAGCAACTGCATAAG-3′) and lucB134 (5′-ATGTTTCAGGTTCAGGGGGAGG-3′).

Quantitative PCR. The DNA samples for PCR assays were also used in quantitative (Q) PCR analyses. Q-PCRs used the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI Prism 7700 Sequence Detector (Applied Biosystems). One Q-PCR assay was designed to amplify 72 bp of luciferase cDNA (from nt 309–381) using the primers lucq1F (5′-GGCGCGTTATTTATCGGAGTT-3′) and lucq2R (5′-TACTGTTGAGCAATTCACGTTCATTAT-3′). The standard for luciferase quantitation was pGL2-basic, described above. A second Q-PCR assay was designed to amplify 85 bp of the rat AQP5 water channel gene (from nt 190–275), using the primers rAQP5q1 (5′-TGGCCTGGGCTCAGCA-3′) and rAQP5q2 (5′-GGCAATTGAGATTTGCAGAATG-3′). The standard for rat AQP5 quantitation was pAQP5 (American Type Culture Collection, Manassas, VA), which includes 1400 bp of rat AQP5 genomic DNA.

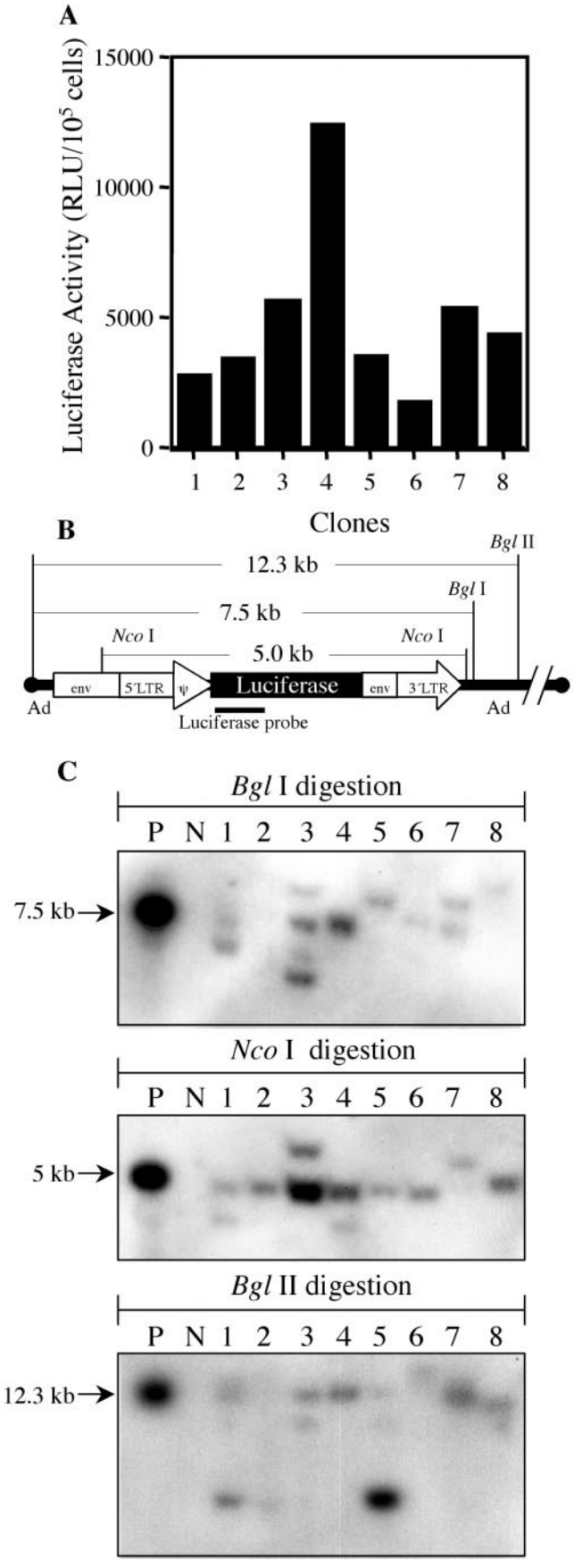
Southern hybridization. Genomic DNA and low molecular weight DNA was extracted as above. Fifteen micrograms of DNA/sample was digested with restriction enzymes and separated on a 1% agarose gel. Nucleic acids were then transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England). Blots were hybridized with a ^{32}P-radiolabeled luciferase probe (a 615 bp *Hin*dIII/*Eco*RI fragment from the 5′ end of the luciferase cDNA) and autoradiographed.

RESULTS AND DISCUSSION

Studies with AdLTR-luc-Infected A5 Cells

Luciferase activity in the eight selected clones is shown in Fig. 2A, 9 months postinfection. Luciferase activity varied over ~6-fold range. Genomic DNA samples were obtained, and Southern hybridization analyses performed after digestion by either *Bgl*I, *Nco*I or *Bg*II restriction endonucleases. The results demonstrate that integration occurred in all eight clones (Fig. 2C). Some clones (e.g., clones 1, 3, 4, 7) exhibited >1 hybridization band suggesting more than one integration event. Hybridization positive bands within individual clones also differed in intensity. These results likely reflect the impurity of the initial cloned cell (only a single dilution step was used).

We extracted high and low molecular weight DNA from all eight A5 clones 6 months postinfection with AdLTR-luc. DNA samples were digested with *Bam*HI and *Not*I (see Fig. 1). Ethidium bromide staining demonstrates that our extraction procedures provide two clearly different DNA samples with respect to size (Fig. 3A). After DNA transfer and probe hybridization we detected the 2.7 kb luciferase cDNA only in the high molecular weight DNA samples (Fig. 3B). The low molecular weight DNA samples, even after film exposure for 2- or 3-fold longer times, showed no evidence for the intact luciferase cDNA. This suggested that no intact AdLTR-luc vector remained in an extrachromosomal location in these A5 clones 6 months after infection. We then examined these DNA samples by a PCR assay (Fig. 3C). All high molecular weight DNA samples showed the appropriate sized (2445 bp) PCR fragment of luciferase cDNA. No PCR products were found in low molecular weight DNA samples from A5 clones unless the film was overexposed (Fig. 3C), and then only A5 clones 1, 7, and 8 showed a weak ~2.5 kb band. This



result indicated that these A5 clones might have trace amounts of intact AdLTR-luc DNA present in an extrachromosomal location.

To further examine this latter possibility, we tested all DNA samples (high and low molecular weight) with a sensitive Q-PCR procedure to measure the presence of the luciferase cDNA. The two luciferase primers used could not amplify any luciferase fragments when genomic DNA samples from noninfected A5 cells were employed (not shown), indicating these two primers were quite specific. In high molecular weight DNA samples there were on average ~270-fold more luciferase copies detected/sample than with low molecular weight DNA samples ($\sim 1.2 \times 10^2 - 2.1 \times 10^3/1.6 \times 10^5$ cells). Luciferase fragments were amplified in all low molecular weight DNA extracts from all eight A5 clones, not only from clones 1, 7, and 8.

As a control for DNA extraction procedures, we examined both DNA fractions from all clones for the presence of the rat aquaporin 5 (AQP5) gene. Since A5 cells are derived from rat submandibular glands, these cells should contain the native AQP5 gene in their genome (16, 17). The 85 bp rat AQP5 genomic DNA fragment also was amplified from both high molecular weight and low molecular weight DNA samples. The presence of detectable rat AQP5 DNA copies in low molecular weight DNA samples indicates that the Hirt extraction method leads to some fragmentation of high molecular weight DNA during the extraction. The ratio of AQP5 molecules in the high to low molecular weight DNA samples (~100–400:1) was similar to that found for luciferase cDNA molecules. These results strongly suggest that the presence of some copies of luciferase cDNA in low molecular weight DNA samples from A5 cell clones is a preparative artifact (i.e., background) and not a reflection of actual intact AdLTR-luc vector DNA presence.

Studies with AdLTR-luc-Infected HSG Cells

The strategy employed for these experiments has its basis in the ability of $E1^-$ recombinant adenoviral vectors to replicate in certain cell lines that provide transcomplementing functions. Any intact replication-

FIG. 2. Infection of A5 cells with AdLTR-luc. (A) Luciferase activity measured in eight A5 clones after 9 months of culture (single measurements, performed monthly; see text for details). (B) A partial diagram of restriction endonuclease digestion sites (*Bgl*I, *Nco*I, and *Bgl*II) at the 5′ end of AdLTR-luc. (C) Southern hybridization analysis testing for integration of the luciferase cDNA in these eight A5 clones. Digestions with the *Bgl*I, *Nco*I, or *Bgl*II restriction endonucleases were performed. The positive control (P) was high molecular weight DNA extracted from uncloned A5 cells infected with AdLTR-luc for 2 days. The negative control (N) was high molecular weight DNA extracted from uninfected A5 cells. The blots shown are representative of two or three performed. A 615 bp fragment of the luciferase cDNA was used to probe blots.

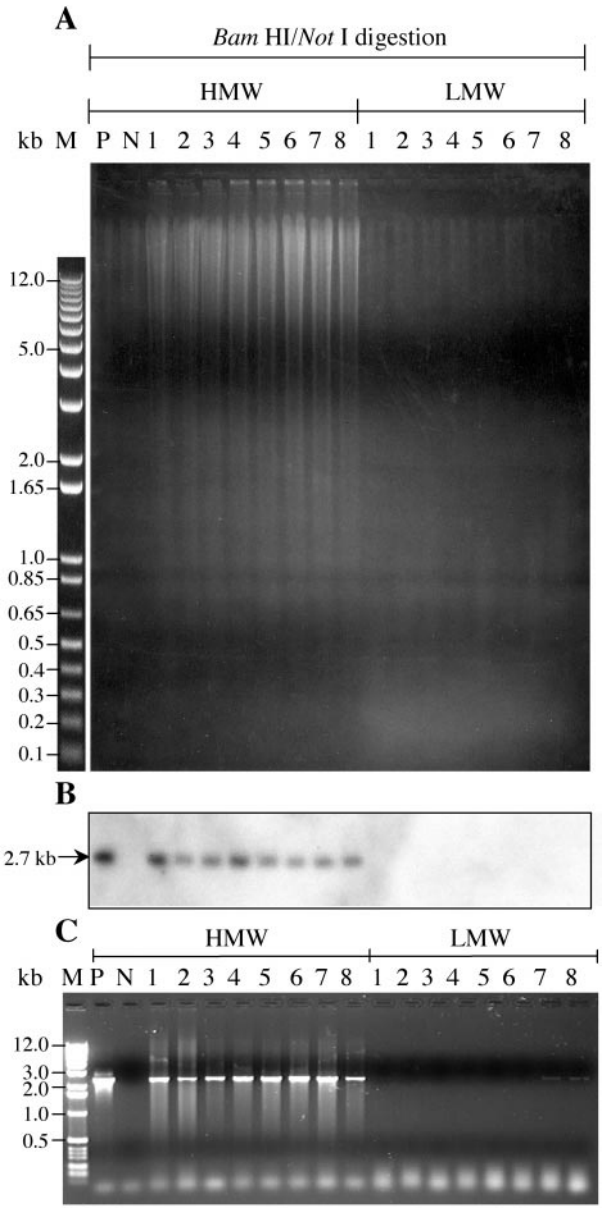


FIG. 3. Examination of the luciferase cDNA in high (HMW) and low (LMW) molecular weight DNA extracts from A5 cell clones. (A) An ethidium bromide-stained gel. (B) Southern hybridization results with the same DNA from A after transfer to a nylon membrane. (C) PCR assay to test for the presence of the luciferase cDNA. This PCR assay was capable of detecting 10 copies of luciferase cDNA. The positive control (P) was high molecular weight DNA extracted from uncloned A5 cells infected with AdLTR-luc for 2 days. The negative control (N) was high molecular weight DNA extracted from uninfected A5 cells. Cloned cell samples are indicated by number. The migration position of standard DNA fragments (M) is shown to the left in A and C. These results are representative of two separate experiments performed.

deficient recombinant adenoviral particle in such cells should replicate and eventually lyse the cells. However, if the adenoviral vector were to mediate integration into the host genome in these cells, in the absence of any extrachromosomal virus, the cells should not lyse but would replicate carrying the foreign genome. The screening of cloned cells for stable transgene ex-

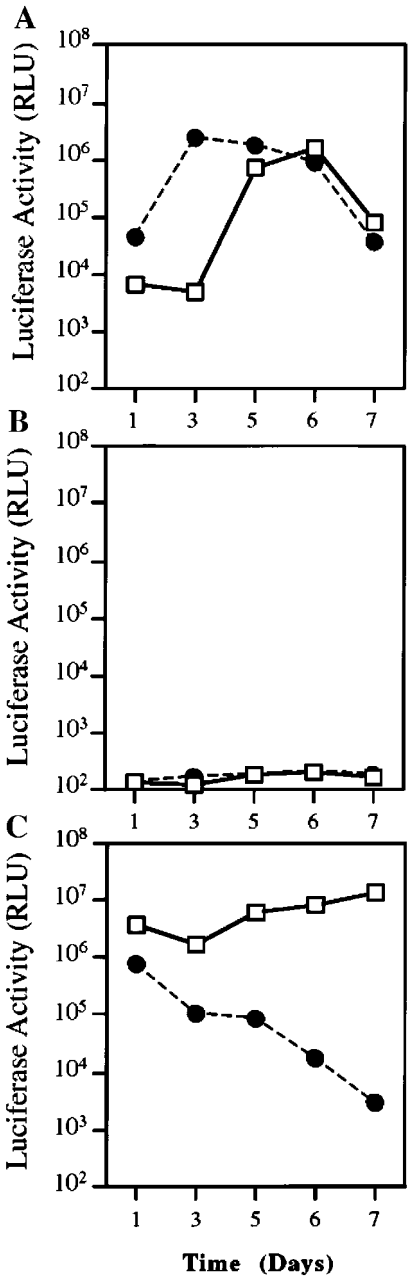


FIG. 4. Determination of the ability of the conventional replication-deficient recombinant adenoviral vector, AdCMV-luc, to replicate in HSG and A5 cells. (A) Luciferase activity found in lysed HSG (□) and A5 (●) cells infected with AdCMV-luc at 1000 particles/cell. The infected cells were harvested at days 1, 3, 5, 6, and 7. Forty microliters of cell lysate on each day was used to test luciferase activity directly (A) or was added to 2 ml growth medium in the absence (B) or presence (C) of 10^5 293 cells. For these last two experiments (B, C), following incubation for 24 h, 40 μl of cell lysate was used to assay luciferase activity present. The results shown are the average of three determinations and are representative of two separate experiments.

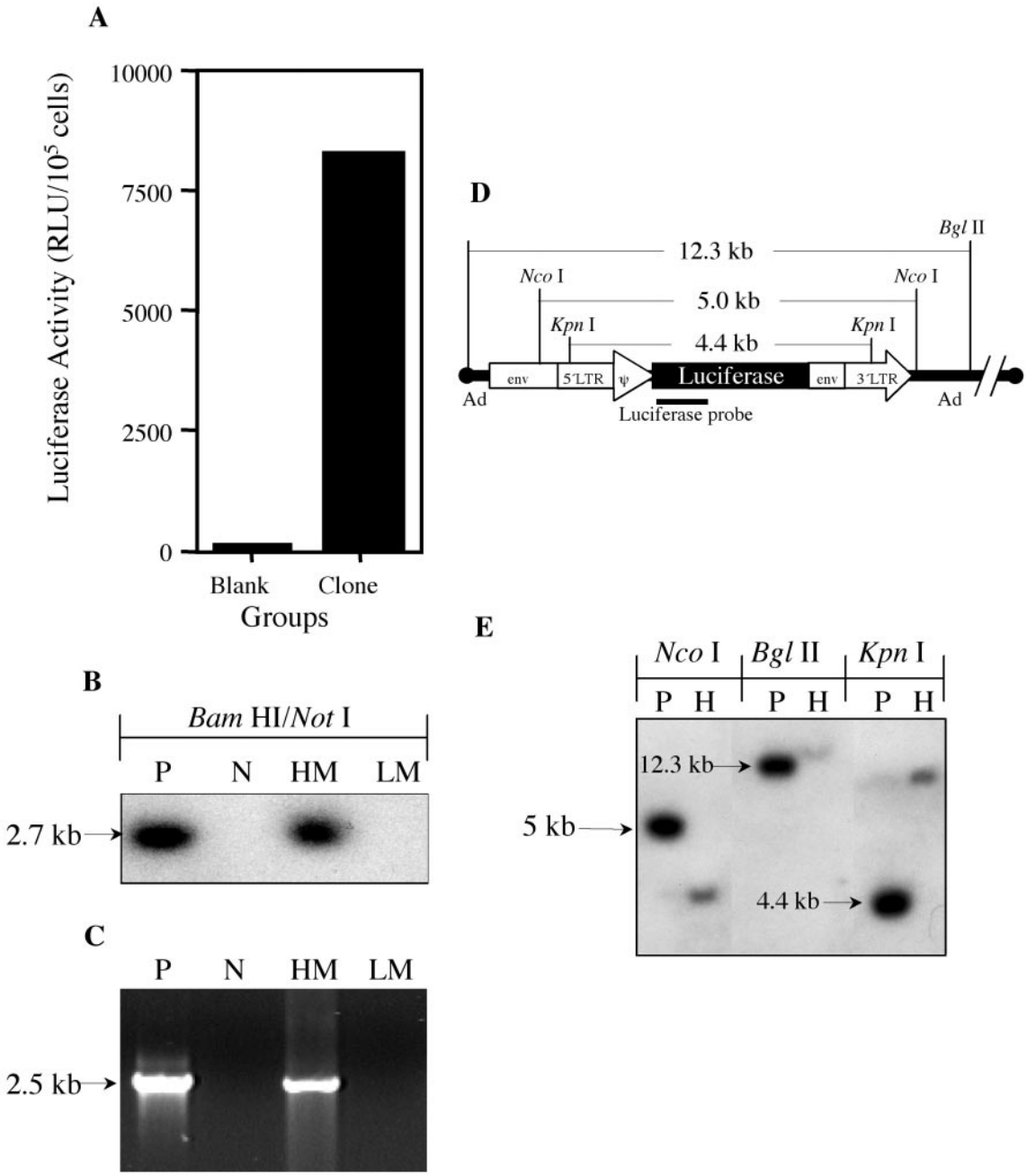


FIG. 5. Examination of luciferase activity and DNA samples from a luciferase-positive HSG cell clone infected 6 months earlier with AdLTR-luc. (A) Luciferase activity measured in a HSG clone after 6 months in culture. Blank is an extract from uninfected HSG cells (single measurements, performed monthly). (B) Southern hybridization. DNA samples were obtained from the HSG clone at 4 months postinfection. Genomic (high molecular weight DNA; HM) and low molecular weight (LM) DNA from this HSG cell clone were digested with *Bam*HI and *Not*I. This result is representative of two separate experiments. (C) PCR analyses of the same DNA samples, as used in B, were carried out as described in the text. This result is of a single experiment. (D) A partial diagram of restriction endonuclease digestion sites (*Nco*I, *Bgl*II, and *Kpn*I) at the 5′ end of AdLTR-luc. (E) Southern hybridization to evaluate luciferase cDNA integration. Only high molecular weight (H) DNA was examined. Samples were digested by either *Nco*I, *Bgl*II, or *Kpn*I and treated as described in Fig. 2. This result is representative of two separate experiments. The positive controls (P) shown in B, C, and E are high molecular weight DNA extracted from uncloned cells infected with AdLTR-luc for 2 days. The negative controls (N) shown in B and C are high molecular weight DNA extracted from uninfected cells.

pression thus provides a convenient indication of foreign gene integration.

To use HSG cells for this purpose it was first necessary to establish that HSG cells could transcomplement E1$^-$ adenoviral vectors. Previous experience indicated that HSG cells are lysed several days postinfection with replication-deficient recombinant adenoviral vectors. We, however, did not know if this effect was

due to direct cytotoxicity (18–20), or caused by adenovirus replication. To determine if adenoviral replication was involved, HSG cells were infected with AdCMV-luc, a conventional adenoviral vector, which exhibits a very low frequency of integration (1). After 7 days, HSG cells exhibited viral dose-dependent levels of cell lysis (data not shown), consistent with the notion that AdCMV-luc was able to replicate in these cells.

Both HSG and A5 cells were next infected with AdCMV-luc at 1000 particles/cell. Infected cells were harvested on days 1, 3, 5, 6, and 7, and lysed by five freeze/thaw cycles. Aliquots of the cell lysate supernatants were used to (i) assay luciferase activity (Fig. 4A); (ii) add to growth medium in the absence of cells (Fig. 4B); or (iii) add to confluent 293 cells (10^5) in growth medium (Fig. 4C). The culture dishes for Figs. 4B and 4C were further incubated for 24 h after which either aliquots of medium without cells (Fig. 4B) or aliquots of the 293 cell lysate (Fig. 4C) was assayed for luciferase activity. Figure 4A shows that luciferase activity in HSG and A5 cells increased to a maximum at days 3–5, and decreased thereafter. Addition of aliquots of HSG or A5 cell lysates to culture medium alone gave only background levels of luciferase activity (Fig. 4B). Conversely, when comparable aliquots of HSG cell lysates were added to 293 cells, considerable luciferase activity was seen after 24 h. The luciferase activity resulting after infecting 293 cells with a 40 μl aliquot of the day seven HSG cell lysate was 3.7-fold higher than that found when the comparable HSG day one cell lysate was used. When this experiment was repeated with lysates from AdCMV-luc infected A5 cells, luciferase activity seen in 293 cells was dramatically decreased from the day 1 to day 7 samples. These experiments are consistent with the idea that AdCMV-luc can replicate slowly in HSG cells, but cannot effectively replicate in A5 cells.

Accordingly, we tested the strategy outlined above by infecting HSG cells with AdLTR-luc at a very low dose (~0.2 particle/cell) for 1-h prior to cloning of cells by a single limiting dilution. At 2 weeks postinfection, only one out of 398 surviving HSG clones exhibited luciferase activity. This clone has continued to exhibit fairly stable luciferase activity for >6 months (last time point checked; Fig. 5A), indicating that the transgene was genomically integrated. This was shown by Southern hybridization and PCR analyses as employed above with A5 clones. High and low molecular weight DNA samples were prepared from the HSG clone and digested with *Bam*HI and *Not* I restriction endonucleases to excise any luciferase cDNA present. Thereafter, Southern analysis was performed on these samples. As is clearly shown (Fig. 5B), the luciferase cDNA could only be detected in the high molecular weight DNA fraction.

We next used the same PCR assay as shown in Fig. 3C, as a more sensitive test for intact luciferase cDNA. Only the high molecular weight DNA fraction yielded the appropriate PCR fragment generation (Fig. 5C). We also used the Q-PCR procedure described above to determine the number of luciferase cDNA copies/cell in the high and low molecular weight DNA samples. In the high molecular fraction there were on average ~4 luciferase DNA copies/cell, while in the low molecular weight DNA fraction there were on average ~0.04 copies/cell. The latter is similar to values seen for the "background," preparatively generated molecules of AQP5 genomic DNA found with low molecular weight DNA samples from A5 cells. Finally, we digested the high molecular weight DNA extract with either *Nco*I, *Bgl*II or *Kpn*I restriction endonucleases, and performed Southern analysis, to directly test for luciferase cDNA integration. As shown in Fig. 5E, after each digest the samples from the HSG cell clone exhibited different hybridization positive band sizes from the positive control. These aggregate data strongly suggest that integration occurred in this HSG clone after infection by AdLTR-luc, and that the luciferase gene expression observed was derived from this integrated transgene.

In conclusion, we used two different experimental strategies to address an important question related to the potential utility of a prototype adenoretroviral gene transfer vector. We asked if following infection by the vector, AdLTR-luc, long-term transgene expression originated from the integrated cDNA or from surviving intact extrachromosomal vector. Our results strongly suggest that the source of long-term expression is from the integrated cDNA.

ACKNOWLEDGMENTS

We are most appreciative of the critical comments on an earlier draft of the manuscript by, and helpful suggestions of, Drs. Robert Kotin (NHLBI, NIH) and Jay Chiorini (NIDCR, NIH). We also thank Drs. Joseph Vitolo and Brij Singh for their reading of an earlier version of the manuscript.

REFERENCES

1. Zheng, C., Baum, B. J., Iadarola, M. J., and O'Connell, B. C. (2000) Genomic integration and gene expression by a modified adenoviral vector. *Nat. Biotech.* **18,** 176–180.
2. Kay, M. A., Glorioso, J. C., and Naldini, L. (2001) Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics. *Nat. Med.* **7,** 33–40.
3. Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000) Molecular structure of genes and chromosomes. *In* Molecular Cell Biology (Lodish, H., *et al.*, Eds.), pp. 294–340,. Freeman, New York.
4. Lever, A. M. (2000) HIV RNA packaging and lentivirus-based vectors. *Adv. Pharmacol.* **48,** 1–28.
5. Chiu, R., and Grandgenett, D. P. (2000) Avian retrovirus DNA internal attachment site requirements for full-site integration *in vitro. J Virol.* **74,** 8292–8298.
6. Harui, A., Suzuki, S., Kochanek, S., and Mitani, K. (1999) Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol.* **73,** 6141–6146.

7. Colicelli, J., and Goff, S. P. (1985) Mutants and pseudorevertants of Moloney murine leukemia virus with alterations at the integration site. *Cell* **42,** 573–580.
8. Panganiban A. T., and Temin, H. M. (1984) The retrovirus pol gene encodes a product required for DNA integration: Identification of a retrovirus int locus. *Proc. Natl. Acad. Sci. USA* **81,** 7885–7889.
9. Boulter, C. A., and Wagner, E. F. (1987) A universal retroviral vector for efficient constitutive expression of exogenous genes. *Nucleic Acids Res.* **15,** 7194.
10. Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) Use of recombinant adenovirus for metabolic engineering of mammalian cells. *In* Methods in Cell Biology (Roth, M. G., Ed.). pp. 161–189, Academic Press, San Diego.
11. Wang, S. L., Baum, B. J., Kagami, H., Zheng, C., O'Connell B. C., and Atkinson, J. C. (1999) Effect of clodronate on macrophage depletion and adenoviral-mediated transgene expression in salivary glands. *J. Oral. Pathol. Med.* **28,** 145–151.
12. Brown, A. M., Rusnock, E. J., Sciubba, J. J., and Baum, B. J. (1989) Establishment and characterization of an epithelial cell line from the rat submandibular gland. *J. Oral. Pathol. Med.* **18,** 206–213.
13. Shirasuna, K., Sato, M., and Miyasaki, T. (1981) A neoplastic epithelial duct cell line established from an irradiated human salivary gland. *Cancer* **48,** 745–752.
14. Graham, F. L., Smiley, J., Russel, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.
15. Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26,** 365–369.
16. He, X., Tse, C. M., Donowitz, M., Alper, S. L., Gabriel, S. E., and Baum, B. J. (1997) Polarized distribution of key membrane transport proteins in the rat submandibular gland. *Pflugers Arch.* **433,** 260–268.
17. Raina, S., Preston, G. M., Guggino, W. B., and Agre, P. (1995) Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *J. Biol. Chem.* **270,** 1908–1912.
18. Zheng, C., Goldsmith, C. M., O'Connell, B. C., and Baum, B. J. (2000) Adenoviral vector cytotoxicity depends in part on the transgene encoded. *Biochem. Biophys. Res. Commun.* **274,** 767–771.
19. Wersto, R. P., Rosenthal, E. R., Seth, P. K., Eissa, N. T., and Donahue, R. E. (1998) Recombinant, replication-defective adenovirus gene transfer vectors induce cell cycle dysregulation and inappropriate expression of cyclin proteins. *J. Virol.* **72,** 9491–9502.
20. Brand, K., Klocke, R., Possling, A., Paul, D., and Strauss, M. (1999) Induction of apoptosis and G2/M arrest by infection with replication-deficient adenovirus at high multiplicity of infection. *Gene Ther.* **6,** 1054–1063.