

Genomic integration and gene expression by a modified adenoviral vector

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A replication-deficient recombinant adenovirus encoding luciferase was constructed using 5' and 3' long terminal repeat (LTR) sequences of the Moloney murine leukemia virus. Gene expression was observed in cultured cells in vitro and in submandibular gland, cortex, and caudate nucleus for as long as three months in vivo. The vector integrated randomly into the genome of both dividing and nondividing cells as determined by fluorescence in situ hybridization (FISH) (10–15% of cells in vitro and 5% in rat spleen in vivo), gene walking, Southern hybridization, and polymerase chain reaction (PCR), in the absence of transcomplementing reverse transcriptase or integrase activity. The new vector combines the high titer and versatility of adenoviral vectors with the long-term gene expression and integration of retroviral vectors.

Key words: gene therapy, adeno-retrovirus vector

Clinical experience suggests that gene therapy has the potential to treat a broad range of both inherited and acquired human diseases, with a low risk of adverse reactions^{1–3}. However, the efficiency of gene transfer and expression in vivo is still disappointingly low². Ideally, a gene therapy vector should efficiently and safely deliver therapeutic genes to the target tissues, and should produce a therapeutic amount of gene product for the appropriate time. Unfortunately, none of the present vector systems meets all of these requirements^{3–5}.

Moloney murine leukemia virus (MoMLV) was one of the first viral vectors used for human gene therapy. Although it is associated with only a minimal safety risk, its low titer and low gene transfer efficiency make it more suitable for ex vivo use. Because the viral preintegration complex can only translocate to the nucleus during cell division, MoMLV integrates into the genome of only dividing cells. Disappearance of the nuclear membrane during mitosis is necessary for import into the nucleus of the viral preintegration complex of MoMLV^{6–8}. The ability of the virus to integrate into the host genome provides the possibility of long-term gene expression.

Adenovirus is also widely used in vivo as a gene transfer vector. In contrast to a retrovirus, the transport of an adenovirus to the nucleus is rapid in both dividing and nondividing cells. Adenoviruses can be produced at very high titers and may infect cells with high efficiency. Adenoviruses integrate into the cell genome only at very low frequency, which results in unstable gene expression^{9–13}.

Recently, several research groups have attempted to construct a hybrid vector that included both retroviral and adenoviral elements^{14–18}. The general strategy adopted in those studies was to use several adenoviral vectors to provide transcomplementing functions able to support the production of a recombinant retroviral vector in vivo. Reports by Feng and colleagues¹⁴ and Caplen and colleagues¹⁵ demonstrated the feasibility of this strategy by showing proviral integration in a nude mouse tumor model system. A drawback of this approach is that cells must be infected by more than one adenovirus. In addition, because the recombinant vector produced in vivo is a retrovirus, cell division is still required for the virus to become integrated. This is a significant drawback to targeting cells that are terminally differentiated and nondividing.

We report the construction of replication-deficient recombinant adenoviral vector (AdLTR-*luc*) carrying the 5' and 3' LTR sequences from MoMLV and the luciferase reporter gene. It was hypothesized that the MoMLV 5' and 3' sequences could facilitate integration of the vector into host genomic DNA and facilitate long-term transgene expression.

Results and discussion

Gene expression in vitro and in vivo. The salivary epithelial cells (HSY, A5, and HSG) grow readily in vitro. Human mononuclear cells, macrophages, and rat hippocampus neurons were cultured without cell proliferation. All of these cell types were readily infected by AdCMV-*luc* and AdLTR-*luc* (Fig. 1A, B). Two replication-deficient recombinant vectors, both based on the adenovirus type 5 genome, were constructed (see Experimental protocol for details). In AdLTR-*luc*, the luciferase gene is expressed from the MoMLV 5' LTR promoter and followed by MoMLV 3' LTR sequences. AdCMV-*luc* is a conventional first generation adenoviral vector in which the luciferase gene is expressed from the cytomegalovirus (CMV) promoter. AdCMV-*luc* contains no MoMLV sequences.

To assess AdLTR-*luc* and AdCMV-*luc* persistence in vivo, rat submandibular glands were infected locally by retrograde ductal instillation of 1×10^9 plaque-forming units (p.f.u.)/gland. Luciferase activity dropped quickly in both virus groups during the two weeks following infection (Fig. 1C). However, luciferase activity was then stabilized in the AdLTR-*luc* infected rats for up to nine weeks (the last time point studied) while it continued to decline in the AdCMV-*luc* group. At nine weeks, average luciferase activity in glands administered AdLTR-*luc* was 15-fold greater than in glands administered AdCMV-*luc* (9.1 relative light units (r.l.u.)/25 μ g protein vs. 0.6 r.l.u.), despite the greater strength of the CMV promoter relative to the LTR¹⁹.

Rat brain was injected using a stereotactic head frame with a total dose of 1×10^8 p.f.u./brain. After three months, AdLTR-*luc* infection led to markedly higher amounts of luciferase in the brain in vivo than the control vector, AdCMV-*luc* (Fig. 1D, E). Luciferase expression in the AdLTR-*luc* infected group did not change appreciably by the fourth

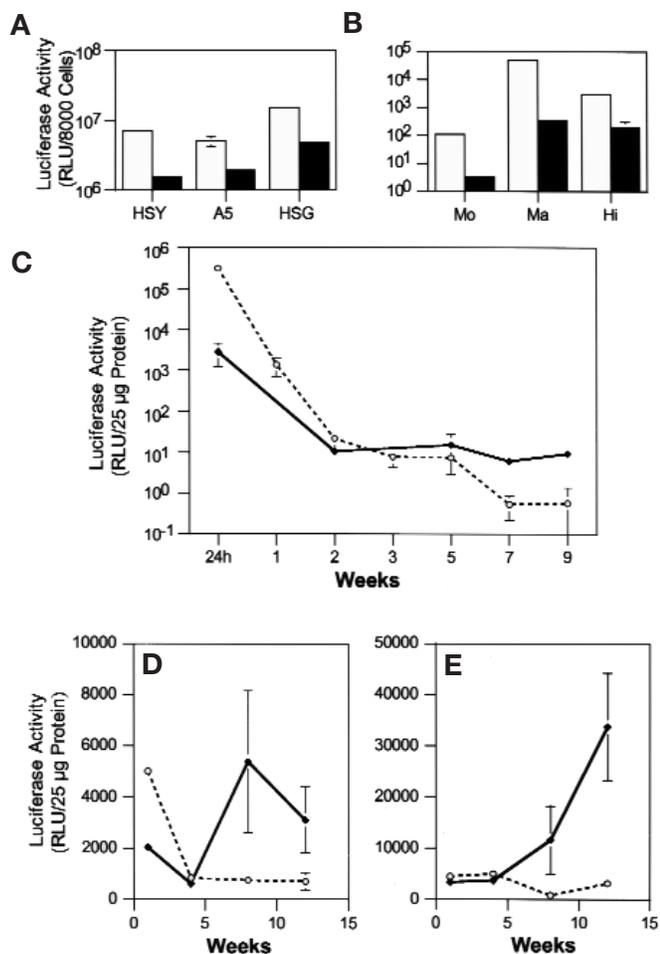


Figure 1. Luciferase expression by AdLTR-luc in quiescent and dividing cells, brain and submandibular gland. The following cells were used in vitro: the salivary epithelial cell lines HSY (human), A5 (rat), HSG (human), mononuclear cells (Mo), and macrophages (Ma) from normal human peripheral blood, hippocampus neurons from rat brain (Hi). Dividing cells (A) or nondividing cells (B) were infected with AdCMV-luc (□) and AdLTR-luc (■). (C) Luciferase activity in rat submandibular gland after retrograde ductal infusion of virus (1×10^9 p.f.u./gland). The amounts of luciferase were measured in the overlying cortex (D) and in the caudate nucleus (E). Rats received either AdCMV-luc (○) or AdLTR-luc (◆). The data are the mean \pm s.d. of three assays for cells in vitro and three samples from three rats in vivo.

week, but increased 6- to 13-fold by weeks 8 and 21 in both the cortex and the caudate nucleus ($\sim 3,101$ and $33,755$ r.l.u./25 μ g protein at 12 weeks, respectively). In contrast, luciferase expression in the AdCMV-luc infected group was initially high in the cortex ($\sim 5,000$ r.l.u./25 μ g protein), but was considerably reduced by eight weeks (~ 700 r.l.u./25 μ g protein) and did not change further. Earlier adenoviral vectors have shown much shorter terms of transgene expression²⁰⁻²⁷.

Analysis of vector integration by PCR. Polymerase chain reaction (PCR) primers were designed from three regions of AdLTR-luc (Fig. 2A). The PCR 1 amplicon was 5' to the canonical breakpoint, AATG, at the beginning of U3 in the 5' LTR of MoMLV. PCR 2 abutted the 3' end of the 5' LTR, and PCR 3 was located within the luciferase gene. Amplification of PCR2 and PCR3, but not PCR1, would suggest breakage of the vector in the 5' LTR. In AdCMV-luc infected cells, PCR 1 and PCR 2 should not amplify because this virus contains no LTR sequence. A5 cells were infected with AdCMV-luc or AdLTR-luc and individual luciferase-expressing clones were isolated. Total cellular DNA was prepared for PCR analysis. All clones infected with AdLTR-luc contained the PCR 2 and

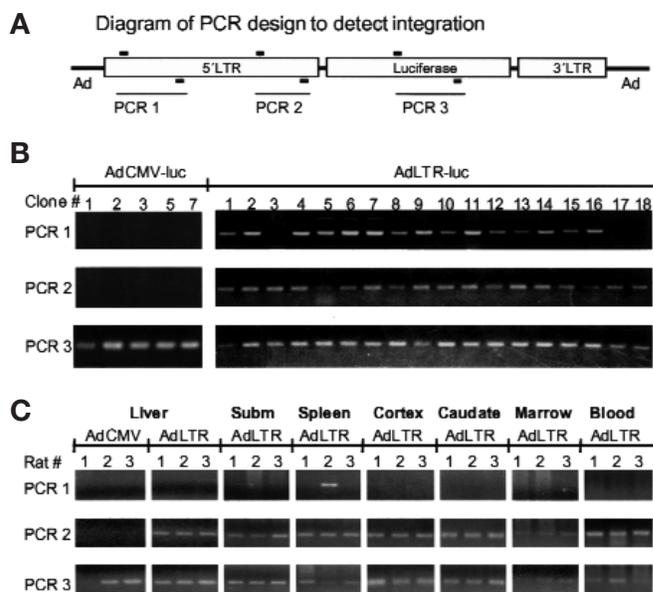


Figure 2. PCR assay for the integration of AdLTR-luc. (A) PCR primers used to detect an intact 5' LTR (PCR 1), the downstream 5' LTR (PCR 2), and the luciferase gene (PCR 3) in AdLTR-luc (AdLTR), or AdCMV-luc (AdCMV). (B) PCR results from cloned A5 cells. (C) Results of the PCR in vivo assays in rats: liver, 12 weeks postinfection; submandibular gland (Subm), 9 weeks postinfection; spleen, 12 weeks postinfection; brain cortex and caudate nucleus, 8 weeks postinfection; bone marrow and peripheral blood, 12 weeks postinfection.

PCR 3 products (Fig. 2B). However, the PCR 1 product could not be found in several clones (3, 17, and 18), suggesting that at least part of the 5' LTR had been lost. Clones yielding a PCR 1 product may represent cells carrying both integrated and episomal vectors. The latter would be detected even if integration occurred. In all, 15.5% of the A5 clones infected with AdLTR-luc alone had lost the PCR 1 amplicon, consistent with the idea that AdLTR-luc could mediate integration in vitro in dividing cells. Similar results were observed in HSY and HSG cells (data not shown).

In vivo, sustained luciferase expression was also related to a loss of the PCR 1 product (Fig. 2C). The PCR 1 product could not be amplified from the livers of any of the three rats that received AdLTR-luc, 8–12 weeks postinfection, from two submandibular glands, two spleens, three brain cortex samples, and three caudate nuclei. In addition, the 5' end of the vector was undetectable in the bone marrow or peripheral blood of the three rats examined at 12 weeks postinfection, although the remainder of the vector seemed intact. Given that most hematopoietic cells have short half-lives, the persistence of vector in bone marrow and peripheral blood suggests stable integration of the vector. The PCR 3 amplicon was detected in all cloned cells and most tissue samples after AdCMV-luc infection.

Determination of vector integration in A5 cells by Southern hybridization. A5 cells do not display any endogenous reverse transcriptase activity (data not shown). Cells were infected with either AdLTR-luc or AdCMV-luc and cloned 10 days postinfection. Luciferase was expressed by 18 out of 22 AdLTR-luc infected cell clones and 16 out of 24 AdCMV-luc infected clones, and genomic DNA from selected clones was digested by several restriction endonucleases and probed with a 615-bp Hind III/EcoR I fragment from the 5' end of the luciferase cDNA (Fig. 3).

Uncut DNA from both AdLTR-luc and AdCMV-luc infected cells gave rise to a single DNA fragment with an estimated length of ~ 37 kb. When DNA was digested with BamHI and NotI, all samples yielded a single primary fragment of ~ 2.7 kb, corresponding to the intact luciferase cDNA. DNA samples were next digested with either

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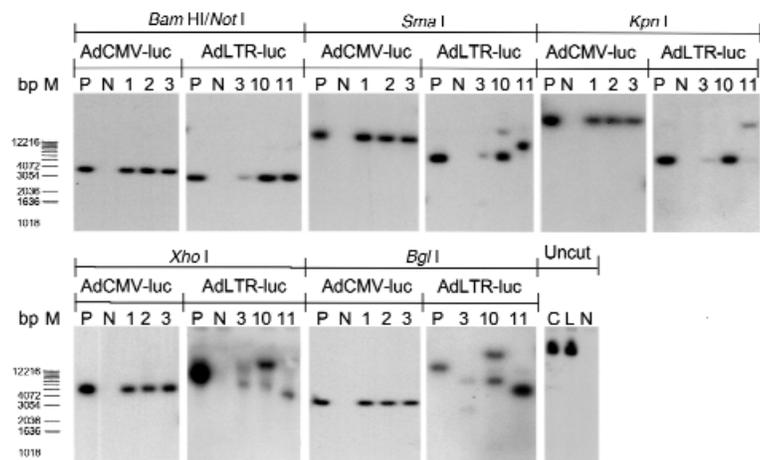


Figure 3. Southern blot analysis for integration of AdLTR-*luc* and AdCMV-*luc* in A5 cells. Positive controls (P) were uncloned cells infected with the indicated virus for two days. N, uninfected cells. Cloned cell samples are indicated by number (for AdCMV-*luc*, 1, 2, 3, and for AdLTR-*luc*, 3, 10, 11). C is AdCMV-*luc* and L is AdLTR-*luc*. Samples were digested with the indicated restriction endonucleases. The migration position of standard DNA fragments (M) is shown to the left.

*Sma*I or *Kpn*I. *Sma*I cleaves once in each LTR and at 13 sites in the adenoviral backbone. There are three *Kpn*I sites in the 5' LTR, one site in the 3' LTR, and 10 in the adenoviral backbone. Neither enzyme cleaves within the luciferase cDNA. DNA from AdCMV-*luc* infected cells showed a single fragment in each clone (Fig. 3). This fragment migrated similarly to the single band seen in the control sample (Fig. 3, P, uncloned AdCMV-*luc* infected cells). Conversely, in DNA samples from cloned AdLTR-*luc* infected cells, fragments of different lengths were detected (P, uncloned AdLTR-*luc* infected cells). After digestion with *Sma*I, larger positive bands (~9 and 6 kb) were detected in clones 10 and 11, whereas after *Kpn*I treatment, clone 11 displayed a larger positive band (more than 12 kb). Finally, DNA samples were treated with either *Xho*I or *Bgl*II (Fig. 3), neither of which cleaves within the LTRs or luciferase cDNA. There are 17 *Bgl*II sites and 6 *Xho*I sites within the adenoviral backbone. Two days postinfection, all samples from AdCMV-*luc* infected cells, including uncloned cells, identified a single fragment (~5 and 4 kb, for *Xho*I and *Bgl*II, respectively). However, the three AdLTR-*luc* clones tested showed fragments with lengths that differed from those detected with control DNA.

These results suggest that the AdLTR-*luc* integrated into genomic DNA and that the vector breakpoints were within the 5' LTR. The *Sma*I site is located at bp 1714 in the 5' LTR, whereas the *Kpn*I sites are at bp 260, 443, and 1720. *Sma*I and *Kpn*I digests suggest that the breakpoint in the 5' LTR is located downstream of bp 1720, a finding consistent with the PCR and gene walking results (below).

Determination of vector integration by FISH. Although no integration of AdCMV-*luc* was detected in infected HSY cells, the frequency of integration by AdLTR-*luc* was estimated to be 15% in both HSY and A5 cells (Fig. 4). HSY cells showed several integrations. Five percent of spleen lymphocytes from intravenously infected rats were also positive for vector integration by FISH (Fig. 4D). To assess the extent of AdLTR-*luc* integration, a two-probe hybridization was carried out. The first probe contained 5' and 3' LTR and luciferase sequences. The second probe was from the E4 region of Ad5 (*Hind*III fragment from bp 30,697 to 36,143 of the plasmid pJM17). These two probes are separated by 30 kb in AdLTR-*luc*. The integrated vector sequences hybridized to the first probe only (Fig. 4E), suggesting that integration occurred between the 5' and 3' LTR sequences. When we exchanged the probe labels and repeated the FISH analysis, we again detected hybridization only to the first probe (not shown).

AdLTR-*luc* integration seemed random, and was observed on chromosomes 22, 8, 10, and 2. It is important to recall that the high frequency of detection of the luciferase gene in cells in vitro by PCR amplicon 3 is probably the result of the presence of nonintegrated, epichromosomally localized virus. The absence of PCR amplicon 1 was noted in A5 cells, however, at a frequency similar to that detected by FISH analysis (~15%).

Sequencing of vector integration site. In the wild-type MoMLV an AATG sequence near the 5' end of the LTR is recognized, two bases (AA) are excised, and the virus integrates at this point²⁸⁻³². Therefore, gene walking was used to sequence the junctions of the presumptive 5' LTR breakpoints in cells infected with AdLTR-*luc* (see Experimental protocol). The antisense primer (A3) was located downstream of the 5' LTR, in the packaging signal sequence of MoMLV (Fig. 5A). DNA was isolated from macrophages, mononuclear cells (both nondividing), and HSY cells (dividing cells) infected with AdLTR-*luc*. Chimeric junction sequences were isolated from all three cell types (Fig. 5B). Several hundred base pairs of 5' LTR sequence were identified, and nonviral sequences including both known (human PAC, human hypocretin (orexin) receptor, and human BAC) and unknown fragments ranging from several base pairs to more than 240 bp were found upstream of the breakpoint. All chimeric junctions found included the potential breakpoint in the 5' LTR between bp 1557 and 1961.

These data confirm that AdLTR-*luc* integrates into the genome of nondividing and dividing cells. The vector was not integrated at the AATG motif, as would be expected for MoMLV²⁸⁻³². The vector sequence pattern at the breakpoints observed was TC, TT, CC, GG, or CT in the macrophages, AC, CC, GA, GC, GT, or CG in the mononuclear cells, and GGG, GGT, GGC, or ACCC in the HSY cells. The heterogeneity of the breakpoints, and the observation here that integration occurred without exogenous integrase, suggests that an atypical process is involved.

Classically^{28,33}, retroviral integration into the host cell genome requires *cis* elements (5' and 3' LTR sequences)^{29,34} and virally encoded integrase³⁴⁻³⁶. However, these requirements may not be absolute. For example, mutations in one terminus of the MoMLV genome did not prevent integration of the viral DNA, albeit in an unusual manner, even though the terminal bases were disrupted²⁹. There are also suggestions that viral DNA can become integrated at low frequency in the absence of integrase^{37,38}. Furthermore, host cellular proteins³⁹⁻⁴¹, as well as the manner in which the target DNA is presented⁴², influence the retroviral integration process. In the present study all of the breakpoints were located in a 404 bp region of the 5' LTR. Adenoviral elements could also contribute to integration.

Adenovirus type 5 integrates only at very low frequency (10^{-3} – 10^{-5} per cell) in vitro¹³. We have shown that a hybrid vector can integrate

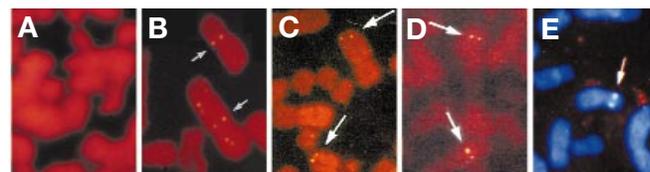


Figure 4. FISH assay for the detection of AdLTR-*luc* mediated integration. (A) HSY cells infected with AdCMV-*luc*, or AdLTR-*luc* (B). (C) A5 cells infected with AdLTR-*luc*. (D) Cells from rat spleen infected with AdLTR-*luc* by intravenous injection. (E) Two-probe hybridization of HSY cells infected with AdLTR-*luc*. Probes are described in the text.

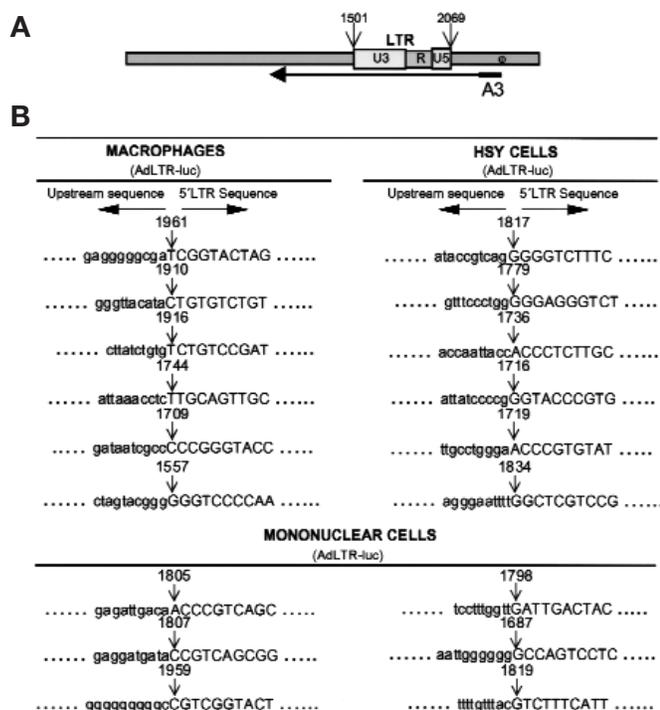


Figure 5. Sequences of vector-genomic DNA junctions. (A) Diagram of gene walking strategy, showing position of the first primer (A3) used for gene walking. The macrophages, mononuclear cells, and HSY cell clones were all infected with AdLTR-luc. (B) Sequences of the junctions.

more frequently when incorporating specific MoMLV LTR sequences. The 5' integration breakpoint is not the classical 5' LTR AATG, and integrase activity was not necessary. Our data were derived from several experimental approaches, all of which strongly support the conclusion that the hybrid vector AdLTR-luc can integrate into genomic DNA much more frequently, and express much longer, than conventional adenoviral vectors.

Experimental protocol

Recombinant viral vectors. The vectors used were based on the adenovirus type 5 (Ad5) genome. E1 deletion was achieved by recombination of the pAC shuttle plasmid with pJM17. A 2.7 kb length of 5' LTR (which includes 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 3' LTR (which contains a small part (~0.5 kb) of the envelope gene and an intact 3' LTR) MoMLV sequences were cleaved by *EcoRI* from the plasmid pXT1 (Stratagene, La Jolla, CA)⁴³. *NotI* linkers were added to both ends of the 5' LTR fragment and *BamHI* linkers were added to both of ends of the 3' LTR fragment. Then, these two fragments were ligated into pAC (with LTR sequences placed in the deleted E1 adenoviral region), an adenoviral shuttle vector (a gift of C. Newgard; University of Texas-Southwestern Medical Center, Dallas, TX). Thus, this construct did not contain any *gag* or *pol* sequences from MoMLV. The luciferase (*luc*) fragment was cleaved from the plasmid pGL2-Basic (Promega, Madison, WI) and ligated between the 5' LTR and 3' LTR sequences to create pACLTR-luc. The luciferase gene was driven by the 5' LTR promoter. The recombinant adenovirus, AdLTR-luc, was generated by homologous recombination of pACLTR-luc with the pBHG10 plasmid in cells from the 293 cell line⁴⁴. AdCMV-luc was constructed as a control vector⁴⁴.

Cell culture. Mononuclear cells and macrophages were obtained from the peripheral blood of normal volunteers. The cells were separated on Ficoll Hypaque, and washed twice with phosphate-buffered saline (PBS). Mononuclear cells were cultured in suspension in RPMI 1640 with 10% human serum for two weeks before infection. Macrophages were adherent to the bottom of the flask after the mononuclear cells from peripheral blood were cultured for a week. The supernatant was replaced by fresh growth medium twice a week for 25 days before infection.

Hippocampus neurons, provided by Z.G. Jiang (NIMH, NIH,

Bethesda, MD), were obtained from Tac:N(SD)fBR rats at 18 gestational days. Hippocampus tissue was cut into 1 mm cubes and triturated by fire-restricted Pasteur pipettes to achieve single cells. Cells were seeded at 40,000/well in a 96-well plate, and cultured in neurobasal medium supplemented with 1×10^{-6} M B27 and 2 mM glutamine for two weeks before infection. This method yields cultures containing 95–98% hippocampal neurons (Z.G. Jiang, personal communication).

The HSY ductal cell line⁴⁵ was obtained from a human parotid adenocarcinoma and was grown in a mixture of 50% Dulbecco's modified Eagles medium (DMEM) and 50% Ham's F12 media. The ductal epithelial A5 cell line⁴⁶ was derived from rat submandibular gland and grown in McCoy's 5A medium. The HSG cell line⁴⁷ was obtained from an irradiated human submandibular gland, and was grown in DMEM/F12 medium. All cells were infected with AdLTR-luc or AdCMV-luc at 50 p.f.u./cell.

Animal experiments. All experimental protocols were approved by the NIDCR Animal Care and Use Committee (ACUC), and the National Institutes of Health (NIH) Biosafety Committee, and procedures were conducted in accordance with the IASP standards for the treatment of rats. Male Wistar rats (250–350 g, three months old) were used for in vivo studies. Rats were anesthetized with ketamine (36 μ g/g body weight) and xylazine (3.2 μ g/g body weight) intraperitoneally, and positioned in a stereotactic head frame. They were then infused over 15 min with 2 μ l of virus (1×10^8 p.f.u./rat) into the caudate nucleus (anteroposterior (AP), +2; mediolateral (ML), +3; dorsoventral (DV), -6.5). At week 1, 4, 8, and 12, the tissues of the caudate nucleus and part of the cerebral cortex overlying the caudate nucleus and encompassing the needle track were collected for the luciferase or PCR assays. For the rat submandibular gland infection, the viruses (1×10^9 p.f.u./gland) were injected by retrograde ductal instillation²⁷. The other tissues were infected by a femoral vein injection of 1×10^9 p.f.u./rat.

Luciferase assay. Cells and tissues were homogenized when necessary, then lysed in cell lysis buffer (Promega) for 15 min. To 100 μ l of luciferase substrate were added 50 μ l of cell lysate, and light output was measured with a luminometer. Results were expressed as relative light units (r.l.u.) per cell number or per microgram protein.

PCR assays. Genomic DNA was extracted with either a Wizard Genomic DNA Purification Kit (Promega) or a Non-Organic DNA Extraction kit (Intergen, Purchase, NY). In each reaction, 200–1,000 ng of template DNA were used. The assay was capable of detecting 10 viral particles for each of the three targets described. The same amount of template DNA was added in all three PCR assays. The primers 5' LTRS2 (5'-TCTCCACCACCATACT-GAACC-3') and 5' LTRA1 (5'-TCAAACACTAGAGCCTGGACC-3') produced PCR 1. PCR 2 was amplified by 5' LTRS4 (5'-TGTGGTCTGGTAGGAGACG-3') and 5' LTRA3 (5'-CCAACGTCTCTTCTTGACAT-3'). PCR 3 is a luciferase product and amplified by lucS2 (5'-AGCGGAATTATGTGTCAGAGG-3') and lucA2 (5'-TTGGGGTGTGTGAACAATA-3'). All negative amplifications were repeated by increasing the amount of template DNA and reamplifying at least twice.

Southern hybridization. The genomic DNA used in the Southern hybridization was extracted with a Non-Organic DNA Extraction kit (Intergen). Twenty micrograms of genomic DNA from each sample were digested with restriction enzymes and separated on a 1% agarose gel. Nucleic acids were then transferred to nylon membranes. The blots were hybridized with a ³²P-radiolabeled luciferase probe (a 615 bp *HindIII/EcoR* I fragment from the 5' end of the luciferase cDNA) and autoradiographed.

FISH detection. HSY cells were cultured for a week after AdLTR-luc infection at 10 p.f.u./cell; A5 cells were cultured for two weeks. Rat spleen was infected with AdLTR-luc at 1×10^9 p.f.u./rat by intravenous injection, and tissue was collected 10 days later. FISH assays were carried out by SeeDNA Biotech, Inc. (Toronto, Canada). Data represent experiments with six separate cell preparations. Probe 1 was pACLTR-luc, which had 5' and 3' LTR and luciferase sequences. This probe was biotinylated with dATP using the BRL BioNick labeling kit. In the two-probe hybridization, the second probe (E4 probe) was obtained from pJM17 between *HindIII* sites 30,697 and 36,143. The E4 probe was digoxigenin labeled with dATP, also using the BRL BioNick labeling kit. As a further control for the labeling conditions, the labels were switched for probe 1 and the E4 probe (i.e., pACLTR-luc was labeled with digoxigenin and the E4 probe with biotin). FISH detection was performed as described⁴⁸.

Sequence of integration sites. The gene walking experiment was carried out by using a 5' RACE (rapid amplification of cloned cells) system (Gibco BRL, Rockville, MD). Two specific antisense primers were used to sequence the junction; 5' LTR A3 (5'-CCAACGTCTCTTCTTGACAT-3') and 5' LTR

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A2 (5'-GAAACACAGTCAGACAGAGA-3'). The primer 5' LTR A3 was used to synthesize a single antisense strand of DNA from the 5' LTR. This single-stranded DNA was tailed with dCTP. Further PCR assays were carried out using 5' LTR A2 and the Abridged Anchor primer (Gibco BRL) and Abridged Universal Amplification primer (Gibco BRL). The PCR products were cloned into the pCR 2.1 plasmid (Invitrogen, Carlsbad, CA), and the positive clones were sequenced.

Acknowledgments

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