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Recombinant Adeno-Associated Virus-Mediated High-Efficiency, Transient Expression of the Murine Cationic Amino Acid Transporter (Ecotropic Retroviral Receptor) Permits Stable Transduction of Human HeLa Cells by Ecotropic Retroviral Vectors

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Adeno-associated virus has a broad host range, is nonpathogenic, and integrates into a preferred location on chromosome 19, features that have fostered development of recombinant adeno-associated viruses (rAAV) as gene transfer vectors for therapeutic applications. We have used an rAAV to transfer and express the murine cationic amino acid transporter which functions as the ecotropic retroviral receptor, thereby rendering human cells conditionally susceptible to infection by an ecotropic retroviral vector. The proportion of human HeLa cells expressing the receptor at 60 h varied as a function of the multiplicity of infection (MOI) with the rAAV. Cells expressing the ecotropic receptor were efficiently transduced with an ecotropic retroviral vector encoding a nucleus-localized form of β-galactosidase. Cells coexpressing the ecotropic receptor and nucleus-localized β-galactosidase were isolated by fluorescence-activated cell sorting, and cell lines were recovered by cloning at limiting dilution. After growth in culture, all clones contained the retroviral vector genome, but fewer than 10% (3 of 47) contained the rAAV genome and continued to express the ecotropic receptor. The ecotropic receptor coding sequences in the rAAV genome were under the control of a tetracycline-modulated promoter. In the presence of tetracycline, receptor expression was low and the proportion of cells transduced by the ecotropic retroviral vector was decreased. Modulation of receptor expression was achieved with both an episomal and an integrated form of the rAAV genome. These data establish that functional gene expression from an rAAV genome can occur transiently without genome integration.

During the past decade, there have been substantial efforts invested in the development of effective strategies to correct genetic defects or to add new functions to cells by gene transfer for therapeutic purposes (11, 13, 19, 42, 44). More than 100 protocols have been approved for the evaluation of gene therapy approaches in humans. Valuable results have already been obtained. For example, gene marking studies in the context of autologous transplantation have documented that neoplastic cells in the bone marrow graft may contribute to relapse in patients with neuroblastoma or acute myeloid leukemia (7, 8, 47); such marking strategies can now be used as tools to evaluate various purging methods. Gene correction of peripheral T lymphocytes (5, 6) or stem cells in bone marrow (6) or umbilical cord blood (30) has been found in patients with severe combined immunodeficiency due to inadequate amounts of the enzyme adenosine deaminase. Despite these promising early results, the development of gene therapy has been limited by the relative inefficiency and unpredictability of the methods used for gene transfer.

Investigators in the field of gene therapy have attempted to exploit the natural capacity of viruses to transfer and express genes in eukaryotic cells, but each of the viruses used to date has limitations. Packaging cell lines have been derived to express the matrix structural proteins (Gag), enzymes (Pol), and envelope proteins (Env) encoded by murine retroviruses that can, upon introduction of a retrovirus genome, generate virions free of replication-competent retroviruses (38, 44). Such vector preparations efficiently transfer genes into proliferating cells in culture, but many important, therapeutic targets, e.g., hematopoietic stem cells, respiratory epithelial cells, hepatocytes, and neurons, are quiescent and therefore refractory to retrovirus-mediated gene transfer (39, 42, 44). Failure to express the protein that acts as a receptor for initiating viral entry may also limit gene transfer, e.g., into primitive hematopoietic cells or hepatocytes (10a, 45). Adenovirus vectors efficiently transfer genes into quiescent cells, although the genome remains episomal and therefore expression is transient (11). The first generation of adenovirus vectors evoke an immune response that destroys cells harboring the vector genome and/or precludes readministration of the vector to achieve a sustained therapeutic effect (62). Nonviral vectors such as liposomes may ultimately prove useful for therapeutic gene transfer but are currently limited by inefficiency and difficulty in achieving reproducible results.

In the context of limitations in the transfer methods available, there has been much interest in the development of

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adeno-associated virus (AAV) as a gene transfer vehicle. This single-stranded DNA virus with a 4,675-nucleotide genome has a protein capsid which is heat stable and resistant to various solvents, detergents, and extremes of pH, thereby facilitating its purification (43). Replication of AAV depends on coinfection with helper DNA virus, e.g., adenovirus or a herpesvirus, which provide functions in *trans* needed for AAV genome replication and encapsidation (4, 43). AAV has a broad host range in that most cultured human cells can readily be infected (34, 43). The wild-type virus integrates into a preferred site on chromosome 19 by a mechanism that appears to depend on the nonstructural proteins (Rep) encoded by the left half of the AAV genome (20, 31–33, 54, 58, 61). Antibodies to AAV can be detected in a high proportion of normal humans, a minority of whom can be shown to harbor the AAV genome in various cell types (19, 43). No human disease has been linked to AAV infection.

Only the 145-nucleotide inverted terminal repeats (ITRs) of the AAV genome are required in *cis* to generate a recombinant AAV (rAAV) vector (53). The Rep and capsid functions provided in *trans* in cells coincidentally infected by adenovirus result in mobilization of the rAAV genome from a plasmid, its replication, and its encapsidation (51, 53). Since there is no overlap between the viral functions required in *cis* and *trans*, i.e., expression of the Rep- and capsid-encoding genes can be achieved without the ITRs, rAAV preparations remain largely free of wild-type AAV (53). To date, most rAAV preparations have been derived by cotransfection of two plasmids, one containing the rAAV genome (vector) and a second containing the AAV genes (Rep and capsid) without ITRs (helper) into cells which are subsequently infected with adenovirus. After cell lysis begins, cells are recovered by centrifugation, fully lysed by repeated freeze-thaw cycles, centrifuged at low speed to remove cellular debris, and heated at 55°C for 1 h to inactivate adenovirus. Such preparations have a relatively low titer and are contaminated with cell constituents and adenovirus. Recently, various technical innovations, including the derivation of packaging cells with integrated helper and vector genomes, that yield vector preparations of higher titer that can be purified by CsCl buoyant density centrifugation have been reported (9, 10, 56).

To date, rAAV constructs have been shown to infect a variety of cultured and primary human cell types both in vitro and in vivo (17, 21, 23, 40, 46, 59, 64). Hematopoietic progenitors, hepatocytes, neurons, and respiratory epithelial cells are among those that have been transduced (17, 29, 31, 44, 60). Initial results suggested that quiescent cells could efficiently express rAAV-encoded genes, but more recent data indicate that cells engaged in DNA synthesis are transduced at a 100-fold-higher frequency than those that are not in cell cycle (46, 49). With selection for rAAV encoding a dominant marker gene, clones that usually contain an unrearranged vector genome stably expressing the transferred gene or genes can be recovered (25, 34, 37, 57). Available evidence also suggests that expression of an rAAV genome can occur transiently after exposure of cells to rAAV without genome integration (18, 37, 39a).

Our experiments were undertaken to evaluate the biological properties of rAAV with respect to efficiency of transduction, potential for transient gene expression, and the frequency of genome integration. Ecotropic retroviruses infect mouse but not human cells because of interspecies polymorphism in the cationic amino acid transporter that acts as a receptor for this class of viruses (1). Using highly purified rAAV vector preparations encoding the ecotropic receptor (EcoRec), we found that human HeLa cells could be transduced at a ratio of viral particles to target cells (multiplicity of infection [MOI]) of approximately 300 to 30,000, leading to functional gene expression. Under these conditions, integration of the rAAV genome occurred infrequently and only at the highest MOI.

Previously, an adenovirus vector was used to transfer the gene for the amphotropic retroviral receptor, thereby increasing its expression and enhancing transducibility of cultured cells (35). Our results establish that an rAAV can be used to transiently render human cells susceptible to infection by a stably integrating retrovirus with ecotropic specificity without integration of the rAAV genome, properties that may be amenable to exploitation for gene therapy applications.

MATERIALS AND METHODS

Cells, retroviral vectors and recombinant plasmids. Mouse NIH 3T3 fibroblasts, human cervical carcinoma (HeLa) cells, and monkey kidney (COS-7) fibroblasts were maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml (D10) at 37°C in a humidified atmosphere containing 5% CO_2. Producer clones for the recombinant retroviruses, Retro-nβGal-NEO^R and Retro-NGFR (Fig. 1), were derived with the GP+E86 ecotropic packaging cell line (36) as previously described (44). Retro-nβGal-NEO^R encodes a nucleus-localized form of β-galactosidase (nβ-Gal), and Retro-NGFR encodes a truncated form of the nerve growth factor receptor (NGFR) having the external and transmembrane domains but lacking the internal kinase domain (27). Cells were grown to confluency, and medium containing retroviral vector particles was collected, filtered through a 0.45-μm-pore-size filter, and frozen at −80°C until needed. The ecotropic Retro-nβGal-NEO^R exhibited a titer of 3×10^5/ml on NIH 3T3 cells after selection for the Neo^r marker, and the ecotropic Retro-NGFR was estimated to have a titer of 5×10^5/ml by assay for surface expression of the NGFR marker 48 h after transduction of 3T3 cells. A clonal cell line, HtTA22, was obtained by introducing the gene for Tet^R-VP16 (22) under the control of the cytomegalovirus (CMV) immediate-early promoter (Fig. 1, pUHD15-1) into HeLa cells by using a puromycin trap strategy by cotransfection with pUHD-puro2. Initially, several clones were screened for high-level, constitutive expression of the chimeric transactivator by using ptet^0-LacZ in a transient assay as previously described (63), and clone HtTA22 was selected for subsequent use.

Functionally relevant segments of various plasmids used in the present study are shown in the diagrams in Fig. 1. Construction of pUHD15-1, pUHD-puro2, and ptet^0-LacZ has been detailed elsewhere (63). pG1nβGalSVNa, used to obtain the cell line that produces Retro-nβGal-NEO^R vector particles, was obtained from Genetic Therapy, Inc. pG1NGFR was used to obtain the producer clone which yields Retro-NGFR vector particles (11a). pSUB201-EcoRec-DHFR, which yields rAAV-EcoRec-DHFR vector particles, was obtained by inserting a DNA fragment containing the EcoRec cDNA (2) under the control of the Tet^0-CMV minimal promoter (22) followed by a simian virus 40 (SV40) polyadenylation signal and the mutant (L22Y) dihydrofolate reductase (DHFR) cDNA (55) driven by the SV40 early promoter and followed by the SV40 polyadenylation signal into pSUB201 (52). Cloning was performed by using standard recombinant DNA techniques (50). DNA transfection and selection of clones were performed by standard procedures and are described in detail elsewhere (63). All plasmids were purified by standard techniques, including two sequential bandings in CsCl gradients (50).

Generation of rAAV particles. To produce rAAV, we used a recently published strategy (9). It consists of coelectroporating a plasmid containing an SV40 origin of replication and encoding AAV Rep and capsid genes (pSV40oriAAV) and a plasmid containing the vector genome into COS-7 cells. Electroporation efficiency (>90%) was monitored in a parallel plate by including an rAAV vector plasmid encoding nβ-Gal (pAAVRnLacZ). The cells were stained for β-Gal activity 48 h later (see below) and analyzed by fluorescence-activating cell sorting (FACS). Electroporated cells were infected with wild-type adenovirus type 5 at an MOI of 10 48 to 72 h after electroporation; 48 to 72 h later, cells were collected, lysed in 0.5% deoxycholate–0.01% trypsin, and banded through three sequential CsCl gradients. Fractions with a density of 1.392 to 1.424 g/ml were pooled and dialyzed against improved modified Eagle's medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulforic acid (HEPES; pH 7.4) and 5% glycerol. After dialysis, preparations were treated at 55°C for 1 h to inactivate any residual adenovirus. Slot blot analysis was performed to determine virus titers (see below). An aliquot of each rAAV preparation was analyzed by electron microscopy after negative staining with 2% phosphotungstic acid on carbon-coated grids (12) to evaluate the preparation for cell debris and adenovirus contamination. Wild-type adenovirus type 5 was propagated in human 293 cells and purified by two sequential rounds of buoyant density ultracentrifugation in CsCl.

Infection protocols. Transduction by rAAV was performed by incubation of target cells in serum-free Dulbecco's modified Eagle's medium (2 ml/100-mm-diameter plate) containing the desired amount of viral preparation in a 5% CO_2

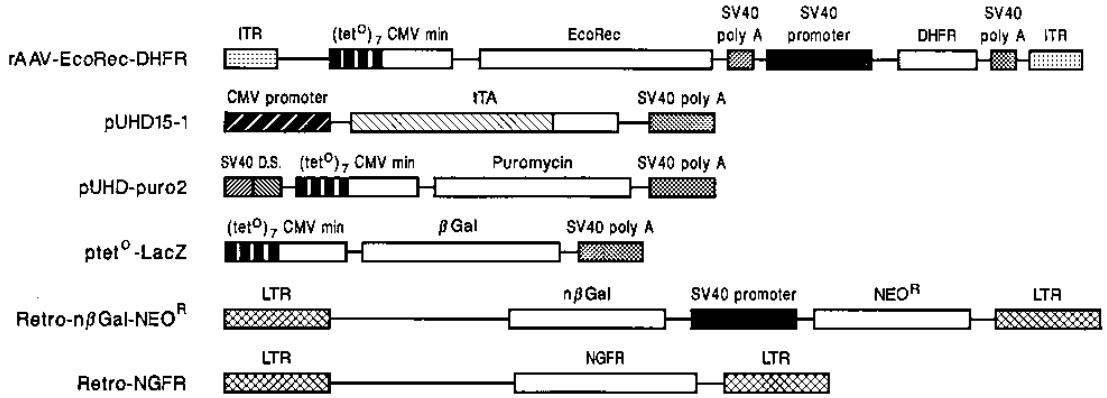

FIG. 1. Organization of expression cassettes and vectors. rAAV-EcoRec-DHFR contains the EcoRec coding sequences under the control of the tetracycline-modulated promoter (tet^0-CMV) and the DHFR (L22Y) coding sequences under the control of the SV40 early promoter. The two expression cassettes are flanked by AAV ITRs. pUHD15-1, pUHD-puro2, and ptet^0-LacZ have been described in detail elsewhere (63). pUHD15-1 encodes the tetracycline-modulated, chimeric transactivator (tTA) (tet^r-VP16) driven by the CMV immediate-early promoter, pUHD-puro2 contains the gene that confers resistance to puromycin under the control of the tet^0-CMV minimal promoter, and ptet^0-LacZ encodes β-Gal under the control of the tet^0-CMV minimal promoter. Retro-nβGal-NEO^R is a retroviral genome encoding nβ-Gal under the control of the long terminal repeat (LTR) promoter and the gene encoding neomycin phosphotransferase, which confers neomycin resistance, under the control of the SV40 early promoter. Retro-NGFR contains the coding sequences for the extracellular and transmembrane domains of NGFR under the control of the LTR promoter. D.S., region from the SV40 genome which contains the polyadenylation signal for both the early and late genes.

humidified atmosphere at 37°C for 2 h with rocking of the plates on a mechanical platform. After 2 h, 8 ml of D10 was added, and the cells were incubated overnight. The next morning, the virus-containing medium was replaced with fresh D10. For retroviral infections, 1 ml of culture medium containing vector particles was added to cells in a final volume of 10 ml/100-mm-diameter plate in the presence of 6 μg of Polybrene per ml and incubated overnight. The next morning, the medium was replaced with fresh D10.

FACS analyses. EcoRec expression was assessed by using a virus binding assay (28). Briefly, 2×10^5 to 5×10^5 cells were incubated with 1 ml of medium containing ecotropic Retro-nβGal-NEO^R particles for 20 min at 37°C in the presence of 6 μg of Polybrene per ml. After two washes with cold PBSA (0.2% bovine serum albumin and 0.2% sodium azide in phosphate-buffered saline [pH 7.4]), cells were incubated for 30 min at 4°C in 50 μl of a sevenfold-concentrated supernatant (83A25) which contains a monoclonal antibody directed against the retroviral ecotropic envelope protein, gp70 (14). Cells were washed and incubated for 30 min at 4°C in 50 μl of PBSA containing 1.5 μg of a fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin antibody (Jackson Immunoresearch). Cells were washed two times with PBSA and analyzed. A control tube in which D10 replaced the vector-containing medium was always analyzed concurrently. To assay β-Gal-positive cells by FACS, fluorescein di-β-D-galactopyranoside (Molecular Probes Inc.) was used as instructed by the manufacturer. Propidium iodide staining was used to exclude dead cells. For NGFR staining, cells were incubated with a monoclonal antibody directed against the extracellular domain of human NGFR (Boehringer Mannheim). Fluorescence was measured with a Becton Dickinson FACS Vantage flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 ± 15 nm. The percentage of positive cells was defined as the fraction with fluorescence intensity equal to or greater than that of 99% of the control cells.

DNA isolation and analyses. Genomic and low-molecular-weight DNA was isolated by using standard protocols (26, 50). DNA was isolated from AAV particles by using proteinase K digestion followed by phenol extraction (53). Southern blot analysis was performed by digesting 10 μg of genomic DNA or 2 μg of low-molecular-weight DNA with one or more restriction enzymes. After size fractionation in a 1% agarose gel, DNA fragments were transferred to a nylon membrane (Hybond N; Amersham) by capillary blotting using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (50). The probes used were a 611-bp Neo^r *Not*I-*Nco*I fragment isolated from pG1Na (Genetic Therapy, Inc.) or a 476-bp *Pst*I-*Hpa*I EcoRec cDNA fragment isolated from pSUB201-EcoRec-DHFR. Probes were labeled by random priming with an oligolabeling kit (Pharmacia) as instructed by the manufacturer. For slot blot analyses, DNA was isolated from AAV particles, and amounts corresponding to 0.08, 0.4, and 2 μl of the viral preparation were denatured in 0.5 N NaOH and spotted onto nylon membranes, using 1 μg of salmon sperm DNA as the carrier. Membranes were hybridized to ^{32}P-labeled probes for wild-type AAV (a 511-bp *Sna*BI-*Hinc*II fragment containing capsid coding sequences isolated from pSUB201), wild-type adenovirus (a 446-bp *Kpn*I fragment isolated from pBSk34k which contains open reading frame 6 from the E4 region), and rAAV (a 476-bp *Pst*I-*Hpa*I fragment of the receptor cDNA obtained from pSUB201-EcoRec-DHFR). As standards for quantitation, serial dilutions of plasmid or adenovirus DNA were spotted in consecutive slots. Quantitation was done with a Phosphorimager.

PCR was performed on 100 ng of DNA. Samples were incubated in the buffer provided by the manufacturer (Promega) at 2 mM Mg^{2+}, 200 μM deoxynucleoside triphosphates, 1 μCi of [α-^{32}P]dCTP, 1 U of *Taq* DNA polymerase, and 75 ng of each primer in a 50-μl reaction. PCR cycling was for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 30 cycles, followed by a 5-min extension period at 72°C. Amplified products were resolved in a 6% polyacrylamide gel, which was subsequently dried and exposed in a Phosphorimager cassette or autoradiographed. The primers used for the EcoRec rAAV genome were 5′ GGAGGC CTATATAAGCAGAGCTCG (sense), which annealed to the Tet^0-CMV minimal promoter, and 5′ GAGAGAATCAGGTTCCAGCCAGTG (antisense), annealing to the 5′ region of the EcoRec cDNA.

RESULTS

rAAV production and characterization. Vector preparations were obtained by the packaging strategy described by Chiorini et al. (9). Serial dilutions of viral DNA were analyzed by slot blot analysis; signal intensities compared with those of serial dilutions of plasmid DNA indicated a particle titer of 10^{10} to 10^{11}/ml. Simultaneous slot blot analysis for the adenovirus or wild-type AAV genome indicated that these were present at a concentration of ≤1 or ≤10% of the rAAV genome, respectively (data not shown). The preparations appeared to be free of cellular debris, and only rare adenovirus particles were identified by electron microscopy prior to heat inactivation (data not shown). All DNA isolated from the vector preparations was resistant to DNase, indicating that it was encapsidated in protein; furthermore, the DNA migrated at a rate consistent with the size of the single-stranded genome (data not shown).

Transduced EcoRec expression in HeLa-derived cells (HtTA22) was directly related to the MOI with rAAV-EcoRec-DHFR (Fig. 2). Low-molecular-weight DNA recovered by the Hirt procedure contained several unidentified molecular species which were resistant to *Dpn*I and sensitive to *Mbo*I, indicating that they had been replicated in eukaryotic cells (data not shown). Very small amounts of DNA with the migration mobility of single-stranded DNA were detected. A small amount of *Dpn*I-resistant DNA having a size of 4.7 kb and comigrating with double-stranded monomer was present (data not shown); this DNA could serve as an episomal substrate for transcription (15, 16). DNA isolated by the Hirt procedure from cells transfected with plasmid DNA was *Dpn*I sensitive. As a further control for the possible contribution of plasmid DNA to the observed results, an unrelated rAAV vector encoding human CD4 to which 20 μg of the plasmid

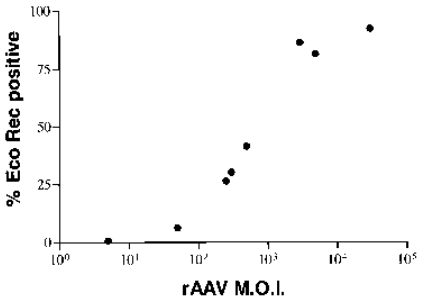

FIG. 2. Determination of rAAV-EcoRec-DHFR titers on HtTA22 cells. Cells (2×10^5) were incubated with increasing amounts of rAAV. EcoRec expression was evaluated 48 h later by staining the cells in the fluorescence binding assay as described in the Materials and Methods. Particle MOI was estimated from slot blot analysis of rAAV DNA. Cells which exhibited a higher fluorescence intensity than 99% of control-stained cells were considered positive. These data suggest that the particle titer exceeded the functional transducing titer in this assay by approximately 1,000-fold.

containing the rAAV-EcoRec-DHFR genome had been added (Fig. 1) was used to transduce HtTA22 cells. No EcoRec expression was observed, whereas expression of the gene encoded in the unrelated rAAV was detected (data not shown).

Efficient transient gene expression by rAAV without genome integration. These experiments were designed to determine whether rAAV-EcoRec-DHFR could be used to facilitate entry of an ecotropic retroviral vector into human cells. HtTA22 cells were exposed to rAAV-EcoRec-DHFR at MOIs ranging from 300 to 30,000. The cells were cultured in the absence of tetracycline to allow full expression of the EcoRec gene. The proportion of cells expressing the EcoRec increased as a function of the MOI (Fig. 3C, E, and G); most cells exposed to an MOI of 30,000 expressed the EcoRec. Twenty-four hours after transfection with rAAV-EcoRec-DHFR, the cells were transduced with Retro-nβGal-NEO^R. When analyzed 36 h later, the proportion of cells expressing nβ-Gal increased as a function of the MOI of rAAV (Fig. 3D, F, and H), presumably reflecting both the proportion of cells expressing the EcoRec and the level of its expression. With this retroviral transduction protocol, the percentage of cells transduced with Retro-nβGal-NEO^R ranged from 15% (Fig. 3D) to 31% (Fig. 3H).

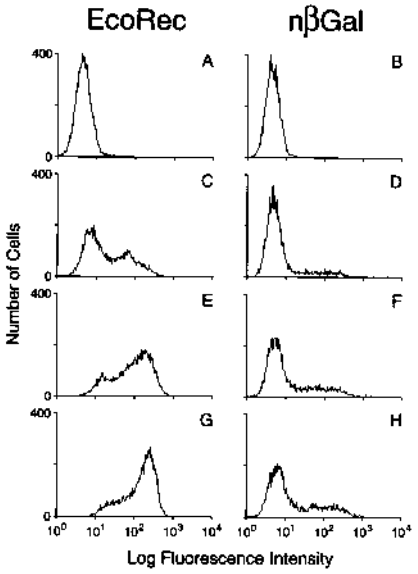

FIG. 3. AAV-mediated, functional EcoRec expression. HtTA22 cells were transduced at MOIs of 300 (C and D), 3,000 (E and F), and 30,000 (G and H) and analyzed for EcoRec expression (C, E, and G) or nβ-Gal activity (D, F, and H) 60 h after exposure to rAAV. Transduction with Retro-nβGal-NEO^R was performed by the standard protocol, beginning 24 h after exposure to rAAV. Panels A and B show results obtained with control cells processed identically but after transduction with a control rAAV at an MOI of 30,000.

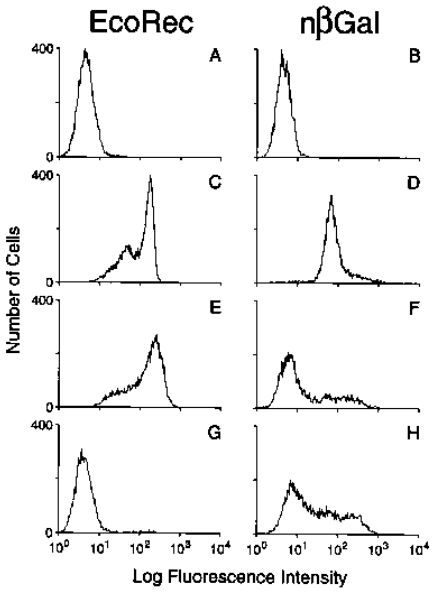

FIG. 4. Loss of rAAV and persistence of retrovirus-mediated gene expression over time. HtTA22 cells were incubated with rAAV-EcoRec-DHFR at an MOI of 30,000 and 24 h later were exposed twice to the ecotropic Retro-nβGal-NEO^R at an estimated MOI of 1 for 12 or 8 h. Fresh medium was added, and 36 h later, the cells were analyzed for EcoRec expression (E) and nβ-Gal activity (F). Cells expressing the β-Gal gene were recovered by FACS, cultured for 17 days, and reanalyzed for EcoRec expression (G) or nβ-Gal activity (H). Panels C and D show positive results with stably transfected cells expressing the EcoRec (C) or nβ-Gal (D). Panels A and B represent negative controls derived by transducing HtTA22 cells with a control rAAV followed by Retro-nβGal-NEO^R and analyzed for EcoRec (A) or nβ-Gal (B) expression.

We next wished to compare the time courses of expression of the genes encoded by the rAAV and retroviral vectors. Cells positive for the nβ-Gal marker were isolated by FACS and passaged for 17 days. These cells continued to express the nβ-Gal gene (Fig. 4H), but only very rare cells expressed the EcoRec (Fig. 4G). Cells expressing the nβ-Gal marker 36 h after transduction (Fig. 3D, F, and H) were also cloned by limiting dilution. After expansion in vitro, DNA from each cell line was found to contain the Retro-nβGal-NEO^R genome by Southern blot analysis (Fig. 5B), whereas only 3 of 47 clones contained the rAAV genome, as determined by PCR (Fig. 5A and data not shown). An additional clone showed a very faint band after prolonged exposure (Fig. 5A, lane 5), probably reflecting an integration event that occurred after the original transduced cell had undergone several cycles of division. Southern blot analysis of DNA from two of the clones that were positive on PCR analyses confirmed integration of the rAAV vector genome (Fig. 6). One clone contained a single integration band, whereas DNA from the other clone contained three, each having an intensity of less than single-copy equivalence, again suggesting that these integration events occurred after the originally transduced cell had divided several times. The three positive clones were derived from HtTA22 cells exposed to rAAV-EcoRec-DHFR at the highest MOI. There was no evidence for selective loss of the integrated

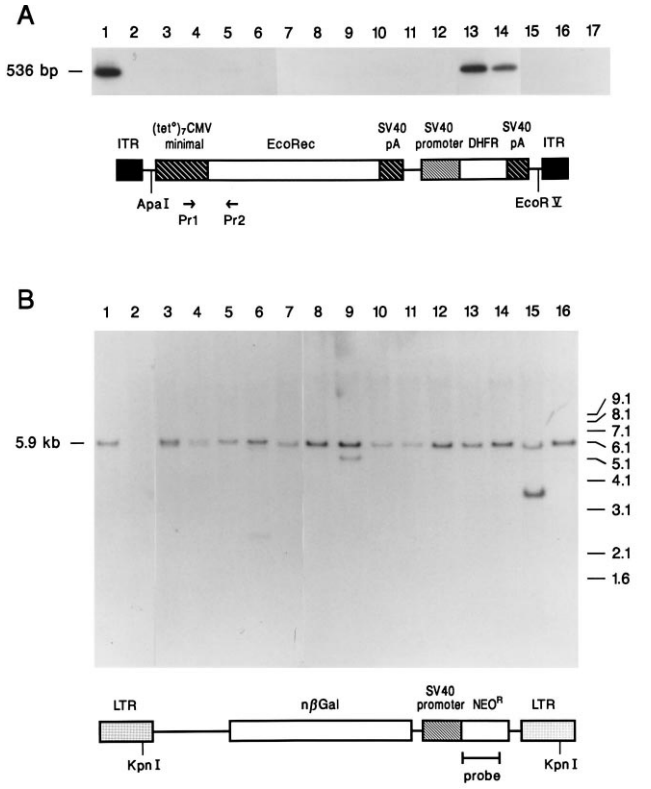

FIG. 5. Integration of the rAAV and retroviral vector genomes. HtTA22 cells expressing the β-Gal gene 60 h after transduction with rAAV-EcoRec-DHFR and 36 h after Retro-nβGal-NEO^R transduction were recovered by cell sorting and plated at limiting dilution. Three weeks later, individual clones were picked; after expansion, genomic DNA was isolated and analyzed by PCR for rAAV sequences (A) or by the Southern blot method for retroviral sequences (B). For the analysis shown in panel B, the DNA was digested with *Kpn*I, which cuts in each long terminal repeat (LTR), releasing a genome-length, proviral fragment. Clones 6, 9, and 15 contain a rearranged retrovirus genome in addition to the full-length genome of 5.9 kb. Lanes 1 and 2 represent positive and negative controls in both panels A and B and correspond to a stably transfected cell line (positive) and parental HtTA22 cells (negative), respectively. DNA from each clone was analyzed for rAAV or retrovirus sequences. Lanes 3 to 12 show corresponding results in both panels; lane 13 in panel A has no corresponding lane in panel B, and lanes 14 to 17 in panel A correspond to lanes 13 to 16 in panel B. Schemes under the panels show the positions of the primers (Pr1 and Pr2) for PCR in the rAAV genome (A) and the expected fragment and probe used in the Southern blot analysis for the retroviral provirus (B).

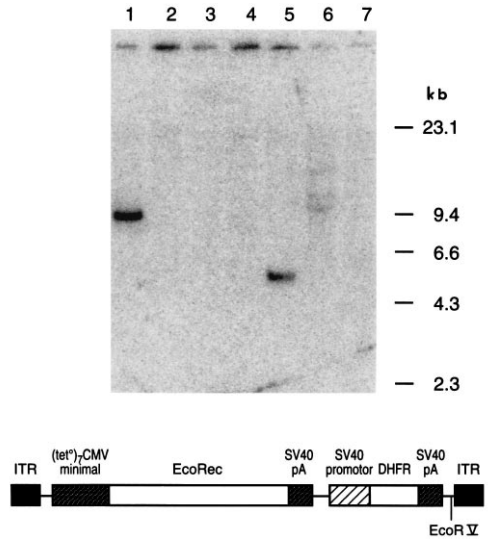

FIG. 6. Integration site analysis of the rAAV genome. DNA from clones that were positive for the rAAV genome on PCR analysis (Fig. 5A, lanes 14 and 15) was analyzed to evaluate integration. DNA was digested with *Eco*RV, which cuts only once in the proviral genome. Lanes 1 and 2 represent positive and negative controls corresponding to genomic DNA from the parental HtTA22 cells to which 30 pg of plasmid DNA containing vector sequences had and had not been added, respectively. Lanes 3 to 7 correspond to lanes 11 to 14 and 5, respectively, in Fig. 5A. The band in lane 5 measured 5.1 kb, significantly longer than the band of 4.4 kb predicted from the unintegrated genome.

rAAV genome, as expressing cells, obtained by transfection with the plasmid containing the rAAV-EcoRec-DHFR genome and isolated by selection in trimetrexate, could be passaged for several weeks in the absence of trimetrexate without loss of expression of the EcoRec. Furthermore, two clones having an integrated rAAV-EcoRec-DHFR genome, as determined by DNA analysis, supported stable expression of the EcoRec over serial passage in culture.

Inducible expression of the EcoRec encoded by an episomal or integrated rAAV genome. The vector used in these experiments was designed so that the level of EcoRec expression could be varied to evaluate the influence of receptor density on infectability with a retroviral vector. A very broad range of receptor density was achieved with the tetracycline-modulated, transactivator/promoter system, as reflected by the data displayed in Fig. 7C (no tetracycline) and Fig. 7E (with tetracycline). These analyses were done 48 h after transduction with rAAV-EcoRec-DHFR, when the genome is likely to be episomal. A severalfold increase in mean receptor expression was accompanied by a modest (7 to 20%) increase in transduction frequency, using a standard transduction assay (Fig. 7D and F).

A population of HtTA22 cells had been isolated by sorting β-Gal-positive cells (Fig. 4H) and recovering individual clones by plating at limiting dilution. One such clone (30.7) was selected for further study. These cells exhibited very low levels of expression of the EcoRec in the presence of tetracycline (Fig. 8E) and minimal infectivity (5%) by the ecotropic retrovirus

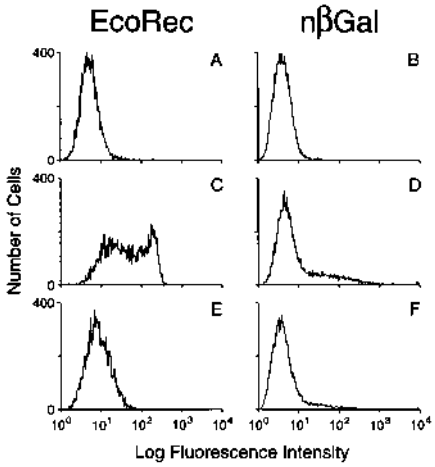

FIG. 7. Modulation of EcoRec expression in cells having an episomal rAAV genome. HtTA22 cells were transduced with rAAV at an MOI of 30,000 in the presence or absence of tetracycline (5 μg/ml). After 48 h, the cells were analyzed for EcoRec expression, and another aliquot was transduced with Retro-nβGal-NEO^R. After an additional 48 h of incubation, the cells were stained for nβ-Gal activity and analyzed by FACS. (A and B) Cells transduced with an unrelated rAAV in the absence of tetracycline; (C to F) cells transduced with rAAV-EcoRec-DHFR in the absence (C and D) or presence (E and F) of tetracycline (5 μg/ml) prior to and during exposure to the ecotropic retroviral vector.

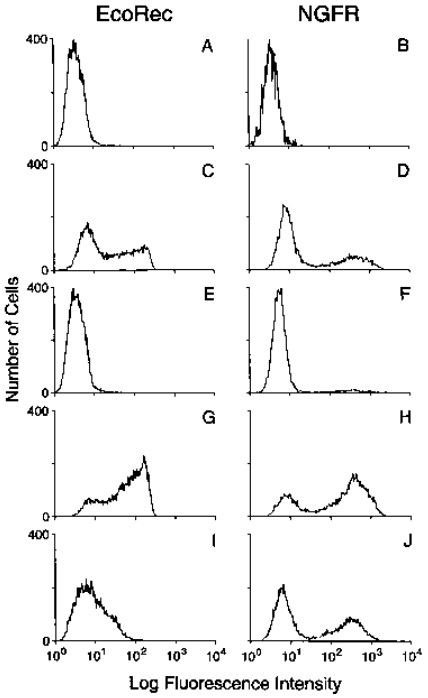

FIG. 8. Modulation of EcoRec expression in cells with an integrated rAAV-EcoRec-DHFR genome. A clone (30.7) having an integrated rAAV-EcoRec-DHFR vector genome (Fig. 6, lane 5) was passaged for 1 week in the absence (C, D, G, and H) or presence (E, F, I, and J) of tetracycline (5 μg/ml). After passage, an aliquot was analyzed for EcoRec expression (C, E, G, and I), and a second aliquot was transduced with ecotropic Retro-NGFR at an MOI of approximately 1. After 48 h, the cells were analyzed for NGFR expression (D, F, H, and J). Panels A and B show the results obtained with parental HtTA22 cells analyzed for EcoRec and NGFR expression, respectively. The cells analyzed in panels G to J were obtained by recovering the most highly positive subset of EcoRec-expressing cells from clone 30.7 ($>10^2$ log fluorescene intensity [C]) by FACS.

(Fig. 8F). In the absence of tetracycline, receptor expression increased variably among the cells that composed the clonal population (Fig. 8C), and retroviral infectivity increased substantially (33%) (Fig. 8D). Integration of the rAAV genome may have occurred one or more cell divisions after the single cell which gave rise to clone 30.7 began to proliferate, resulting in heterogeneity with respect to receptor expression. The subset of cells with highest receptor expression was recovered by sorting and expanded in culture. In the absence of tetracycline, receptor expression was higher than in the unsorted population, and a greater proportion of cells were positive (compare Fig. 8G and C). Basal expression of the receptor persisted in the presence of tetracycline (Fig. 8I). Infectivity by the ecotropic retrovirus encoding NGFR was significant (42%) in the presence of tetracycline (Fig. 8J) and increased to 72% in its absence (Fig. 8H), using the standard transduction protocol. These data establish that the tetracycline-modulated transactivator/promoter system can function in the context of the integrated rAAV genome. Thus modulation of receptor expression was achieved with an episomal (Fig. 7) or integrated (Fig. 8) form of the rAAV-EcoRec-DHFR genome.

DISCUSSION

Our results established that rAAV can transiently express a gene in transduced, proliferating cells without genome integration. Functional expression of the coding sequences for the murine cationic amino acid transporter, the receptor for murine retroviruses with an ecotropic host range, in human cells permitted transduction of these cells with an ecotropic retroviral vector. The tetracycline-modulated promoter system was functional within both an episomal and an integrated form of the rAAV genome. Infectivity of the target population with the ecotropic retroviral vector increased as a function of receptor number.

Wild-type AAV persists in an integrated proviral form in latently infected cells (32, 33, 43, 53, 54), and rAAV constructs encoding a drug resistance marker are demonstrably integrated in drug-selected clones or populations of cells (25, 34, 37, 57, 59). However, without selection, rAAV may be present as an episomal or integrated provirus. Our data and the results of others (15–18, 37) establish that expression of the episomal form may be significant, albeit transient, and that expression within the first several days following transduction cannot be equated with genome integration.

There are at least three separable processes that characterize the interaction of rAAV with target cells: (i) virus uptake and uncoating of the genome, (ii) conversion of the single-stranded genome into a double-stranded form that is a potential substrate for transcription, and (iii) integration of the proviral genome, thereby creating a stable template for persistent gene expression. Various parameters that influence transducibility of target cells, e.g., cell cycle status (18, 46, 49), primary versus established cell lines (23), DNA synthesis inhibitors (48), or DNA-damaging agents (3, 15), could influence any one or more of these processes. Often, there are insufficient data available to distinguish which step or steps are being affected by these parameters.

Available evidence suggests that many cell types are able to absorb and internalize AAV particles fairly effectively, as reflected by the presence of the viral genome in Hirt-extracted DNA within 24 to 72 h after exposure to viral particles (15, 16, 23). The receptor for AAV has yet to be fully characterized. A recent Western blot (immunoblot) analysis identified a 150-kDa binding protein, and the results of a binding assay suggested that receptor number may vary significantly among cell types (41). Indeed we find that an MOI of 1,000 to 3,000 vector particles per cell is sufficient to achieve gene expression in the majority of HeLa-derived cells (Fig. 3), whereas a 4-log-unit-greater MOI is required to achieve gene expression in human erythroleukemia cells or primary progenitor-derived hematopoietic colonies (24). There is a rough inverse correlation between receptor numbers on these cell types, as estimated by the binding assay (41) and the MOI required to achieve gene expression.

Once internalized, the single-stranded rAAV viral genome must be converted to a double-stranded form before it becomes a substrate for transcription. Since both strands of viral DNA are packaged in separate virions, formation of double-stranded DNA could occur by annealing once the genome is uncoated within the target cell, particularly at high MOIs. Alternatively, DNA synthesis could lead to formation of the double-stranded form. Recent data have established a correlation between the amount of double-stranded, episomal genome and the level of rAAV-encoded gene expression during the first 1 to 3 days after exposure of target cells to rAAV (15, 16). Conversion of the single-stranded rAAV genome into its double-stranded form is enhanced by adenovirus gene products encoded by the E1 and E4 (open reading frame 6) regions (15, 16). These adenovirus proteins are thought to enhance expression of cellular genes that facilitate DNA synthesis. The relative ability to convert the single-stranded AAV genome to its double-stranded form could account for the greater transducibility of established cell lines versus primary cells (23) or

cycling versus quiescent cells (49) by rAAV, since these results are based on early gene expression that may reflect transcription of an episomal genome. Similarly, the effects of DNA synthesis inhibitors (48) and DNA-damaging agents (3) may trigger DNA repair mechanisms that enhance transcription of an rAAV genome by facilitating its conversion to the double-stranded form (15).

Our data suggest that genome integration is not the usual outcome after early rAAV-mediated gene expression. We and others (37) have observed that the rAAV genome may persist for several days or even weeks in dividing cells. The half-life of rAAV encoded mRNA and protein may also influence the duration of the biological effect of rAAV transduction. For example, resistance to the G418 analog (37) or the presence of an encoded mRNA species (40) need not reflect genome integration. Only DNA analysis such as Southern blotting to detect a junction fragment or fluorescence in situ hybridization to define chromosomal localization can provide unequivocal evidence of integration of an rAAV genome.

Little is known about the factors that influence rAAV genome integration. The Rep protein of wild-type AAV is thought to facilitate site-specific integration through sequence-specific DNA binding (20, 58, 61), but its effects on the overall efficiency of integration remain uncertain. Some of the parameters that enhance transduction as reflected by early gene expression, e.g., DNA synthesis inhibitors (3, 48) and cell cycle status (49), might also influence rAAV genome integration. Our data suggest that increased MOI enhances integration, but it is difficult to exclude an effect of trace contaminations of adenovirus or wild-type AAV proteins at higher MOIs.

Our experiments suggest that rAAV might be useful for achieving transient expression of a gene product in a target population. Indeed, the ability to express the EcoRec may usefully extend the host range of ecotropic retroviral vectors to human cells without having to rely on the presence or level of expression of the receptor for amphotropic vector particles whose normal host range includes human cells. An element of safety is added since infectivity by the ecotropic virus is temporary and limited to cells transduced by rAAV. The ability to use an inducible promoter system adds another element of flexibility, although in the future, it may be useful to incorporate the gene for the chimeric transactivator into the vector genome. Although the frequency of transduction increased at higher levels of receptor expression, even relatively low levels of receptor expression permitted transduction of a significant proportion of cells (Fig. 8I and J). Additional work will be needed to define any limitations and evaluate the usefulness of rAAV for facilitating retrovirus-mediated gene transfer for therapeutic purposes.

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