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Adeno-Associated Virus Vectors Transduce Primary Cells Much Less Efficiently than Immortalized Cells

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Immortalized cell lines have been used to study infection and replication of adeno-associated virus (AAV) in culture, but primary cells presumably provide a better model for AAV behavior in animals. Here, we have evaluated the ability of AAV vectors to transduce primary and immortalized strains of human epithelial cells and fibroblasts. Two AAV vectors were used, one that transduced an alkaline phosphatase gene (AAV-LAPSN), and one that transduced a β-galactosidase/neomycin phosphotransferase fusion gene (AAV-Lβgeo). The transduction efficiency of the AAV-LAPSN vector, quantitated by measurement of alkaline phosphatase-positive cell foci following infection, was 10 to 60 times greater in immortalized human cells than in primary cells, and total alkaline phosphatase activity in cell lysates was 40 to 50 times greater in immortalized cells. The AAV-Lβgeo vector gave similar results. In contrast, the transduction efficiency of a retrovirus vector encoding alkaline phosphatase was equivalent in primary and immortalized cells. Analysis of the quantity and state of the AAV vector genomes in cells showed that primary and immortalized cells contained comparable numbers of vector copies per cell and that the vast majority of vector DNA was not integrated into the cell genome. Additionally, the level of AAV vector-derived message paralleled the transduction efficiency. These results indicate that the block to functional transduction in primary cells occurred after virus entry and limited the abundance of vector-derived message. Data from AAV transduction in cultures of human cells containing immortalizing genes suggest that cellular changes secondary to the introduction of immortalizing genes increased permissiveness for transduction by AAV vectors. In summary, our data demonstrate that AAV vectors transduce primary human cells much less efficiently than immortalized cells and indicate the importance of using primary cells to evaluate AAV vectors for gene therapy applications.

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus having a genome size of about 4.7 kb. It is nonpathogenic and depends on helper functions provided by adenovirus or herpesvirus for efficient replication and expression (26). In the absence of helper virus, AAV integrates into host chromosomes (26). The isolation of infectious AAV plasmids (31) has allowed the construction of AAV-based vectors. The ability of AAV vectors to effect stable integration (16, 20) makes them attractive as a vehicle for gene delivery and human gene therapy.

Other viral vectors currently being used in gene therapy applications include those based on retroviruses and adenoviruses. The introduction of genes by using retroviruses generally results in efficient gene transfer; however, retrovirus vectors require cell division for efficient transduction (25). Gene transfer with a replication-defective adenovirus vector is efficient (29, 35), but long-term correction with currently available vectors may not be possible because the adenovirus genome exists as an episome in the host cell and therefore can be lost with time. Additionally, adenovirus vectors can express viral late genes, which may lead to cytotoxicity and elicit host immune responses (34).

AAV vectors have some of the positive features found in adenovirus- and retrovirus-based vectors. As in the retrovirus vectors, all viral coding sequences can be deleted from AAV vectors to reduce the possibility of deleterious effects caused by expression of viral proteins. Like adenovirus, AAV is tropic for the upper respiratory tract, supporting the use of this vector system for gene delivery to the airway. An AAV vector carrying the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) has been shown to correct the CF defect in a bronchial cell line (IB3) derived from a CF patient (12) and has been shown to express CF protein following in vivo delivery to the rabbit airway (11). These results suggest that AAV has the potential for use as a virus vector for CF gene therapy; however, the transduction efficiency of this system has not been rigorously evaluated.

Studies of the transduction efficiency of AAV vectors in human cells have generally used immortalized cell lines. In the present study, we used primary human cells to evaluate the transduction efficiency of AAV vectors, based on the premise that results obtained in primary cells would provide a more meaningful prediction of performance in vivo. We found that the transduction efficiency of AAV vectors was much lower in primary airway epithelial cells than in an immortalized airway epithelial cell line. Similar results were obtained in a comparison of transduction efficiency in primary and immortalized human fibroblasts. Experiments were conducted to determine the basis for the different transduction efficiencies in primary and immortalized cells.

MATERIALS AND METHODS

Cell culture. Primary cells from nasal polyps of CF patients were isolated as follows. Polyp biopsy samples were washed extensively, minced into 1-mm squares, and digested overnight at 4°C with Dispase (4 μg/ml; Boehringer Mann-

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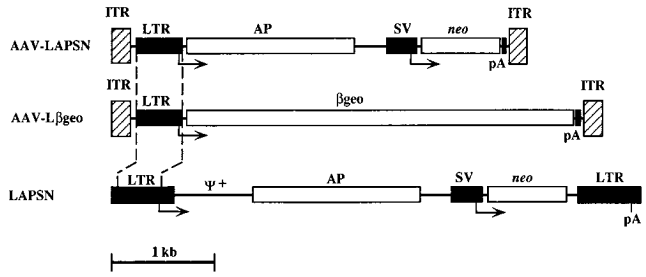

FIG. 1. Viral vectors. Arrows indicate promoters, open boxes indicate coding regions, and dotted lines indicate the region of the retroviral long terminal repeat (LTR) that is present in the AAV vectors. Abbreviations: ITR, AAV inverted terminal repeat; LTR, retroviral long terminal repeat; AP, alkaline phosphatase; SV, SV40 early promoter; *neo*, neomycin phosphotransferase; βgeo, β-galactosidase/*neo* fusion; pA, polyadenylation signal; ψ^+, extended retroviral packaging signal. The sequences between the AP coding region and the SV40 promoter in AAV-LAPSN and LAPSN are 3′ noncoding sequences from the AP cDNA.

heim) in keratinocyte serum-free medium (SFM) (Gibco BRL). The specimens were washed in phosphate-buffered saline and placed in SFM containing 0.1% trypsin and 0.02% EDTA, and then the epithelial sheets were removed by repeated pipetting for 10 min with a Pasteur pipette. The trypsin was inactivated by the addition of 10% fetal bovine serum (FBS). Large tissue fragments primarily containing fibroblasts were allowed to settle, and single cells and cell clusters consisting of epithelial cells were harvested and pelleted by centrifugation. Epithelial cells were cultivated in SFM containing penicillin and streptomycin and used within three passages of initial isolation.

Primary human fibroblasts were isolated from neonatal foreskin after removal of the epithelial sheet by treatment with Dispase. The dermis was minced into 1-mm squares and incubated overnight at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin, and streptomycin and containing collagenase (200 U/ml; Worthington Biochemical). Cells were pelleted by centrifugation and cultivated in Dulbecco's modified Eagle's medium with 10% FBS, penicillin, streptomycin, and amphotericin B.

The IB3 cells, obtained from P. A. Zeitlin (Johns Hopkins, Baltimore, Md.), are human bronchial epithelial cells derived from a CF patient that have been immortalized by an adenovirus-simian virus 40 (SV40) chimeric virus (36). IB3 cells were grown in SFM supplemented with 1% FBS, penicillin, and streptomycin.

LNSV cells ($HPRT^-$ Lesch-Nyhan skin fibroblasts that have been transformed by SV40 [2]); 293 cells (14), used to produce the AAV vectors; PA317 packaging cells (24), used to produce the retrovirus vectors; and HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS, penicillin, and streptomycin.

Introduction of immortalizing genes into primary cells. Primary nasal polyp epithelial cells were plated at 10^6 cells per 10-cm dish on day 1. Human papillomavirus (HPV) type 16 immortalizing genes were introduced on day 2 by exposing the cells to the retrovirus vector LXSN16E6E7, produced by using PA317 retrovirus packaging cells (15). On day 3, cells were trypsinized and split at a ratio of 1:3 into SFM containing 50 μg of G418 (active) per ml. Cells were selected in G418 for 7 days, and polyclonal populations were used for transduction experiments within two passages after selection. Introduction of HPV immortalizing genes into primary human fibroblasts was done in a similar manner except that cells were selected in medium containing 500 μg of G418 (active) per ml.

An SV40 large T antigen gene was introduced into primary human fibroblasts as follows. Psi2 packaging cells producing the retroviral vector Ziptex (7), encoding the SV40 large T antigen, were obtained from P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, Wash.). Virus from these cells was used to infect PA317 cells (24), and the cells were selected in G418 to generate a cell line that produced the Ziptex vector with an amphotropic host range suitable for infection of human cells. Amphotropic Ziptex vector stocks were then used to transduce primary human fibroblasts as described above. Polyclonal G418-resistant cell populations were used for AAV vector transduction experiments.

AAV vector construction and production. The plasmids (pALAPSN and pALβgeo) that were used in the generation of AAV-LAPSN and AAV-Lβgeo viruses (Fig. 1) have been described previously (1, 30). AAV-LAPSN transfers the human placental alkaline phosphatase (AP) gene and the neomycin phosphotransferase (*neo*) gene, and AAV-Lβgeo transfers the fusion gene encoding both β-galactosidase (β-Gal) activity and neomycin phosphotransferase activity (βgeo). Plasmids were propagated in bacterial strain JC8111 (5), and vector stocks were generated as previously described (30). For some experiments, cell lysate prepared following three rounds of freeze-thawing of cell pellets was used, while in other experiments, AAV virions were purified from cell lysates by pelleting the virions through a 40% sucrose (in phosphate-buffered saline) cushion.

Vector stocks were assigned an infectious titer based on their ability to transduce HeLa cells to G418 resistance. HeLa cells were plated at a density of 5×10^5 in 6-cm plates and, 24 h later, infected with 10 μl of vector stock. After 24 h, the cultures were trypsinized and split 1:20 and 1:100 into 10-cm plates. After an additional 48 h, G418 (active) was added at a concentration of 1 mg/ml. Selection was maintained for 10 days, with the medium being replaced on the fourth day. All cells in control cultures not exposed to vector were dead by the seventh day of selection. Colonies were fixed and stained with methanol and Coomassie blue and counted, and the CFU per milliliter of vector stock was calculated. The multiplicity of infection indicated in the experiments is based on the titer determined in this assay. Vector particle concentrations, determined by Southern analysis as described previously (30, 32), were more than 1,000-fold greater than the vector titer in HeLa cells.

Transduction of epithelial cells by AAV vectors. Epithelial cells were plated at 2×10^5 per 6-cm dish and infected the following day with AAV-LAPSN virus at a multiplicity of infection of 0.1, based on the vector titer assayed on HeLa cells. Virus was removed after overnight exposure, and the cells were rinsed in phosphate-buffered saline and refed with SFM. Two days after infection, the cells were processed for AP staining or measurement of AP activity. Infection of epithelial cells by AAV-Lβgeo was done at a multiplicity of infection of approximately 0.01, based on the HeLa cell transduction assay. Two days after virus exposure, the cells were processed for β-Gal staining.

Transduction of fibroblasts by AAV vectors. Primary and immortalized human fibroblasts were plated at a density of 2×10^5 to 5×10^5 cells per 6-cm dish. One day later, the cells were infected with AAV-LAPSN at a multiplicity of infection of 0.001 to 0.01, based on vector titer assayed on HeLa cells. Transduction of AP activity was examined 2 to 3 days postinfection by histochemical staining for AP and by measurement of AP activity in cell lysates.

Histochemical staining. Cells were fixed in phosphate-buffered saline containing 3.7% formaldehyde for 15 min and then washed three times (15 min each) in phosphate-buffered saline. For the AP stain, the cells were incubated at 65°C for 30 min (to eliminate endogenous AP activity) and incubated overnight at room temperature in 100 mM Tris (pH 8.5)–100 mM NaCl–50 mM $MgCl_2$–1 mg of nitro blue tetrazolium per ml–0.1 mg of 5-bromo-4-chloro-3-indolylphosphate per ml. Cells were stained for β-Gal expression after fixation by overnight incubation at 37°C in 25 mM $K_4Fe(CN)_6$–25 mM $K_3Fe(CN)_6$–2 mM $MgCl_2$–1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml in phosphate-buffered saline.

Assay of AP activity in cell lysates. Cell lysates were prepared as described before (4). AP activity in the lysate was determined by a spectrophotometric assay (3), with minor modifications. From 10 to 100 μl of lysate was made up to a final volume of 100 μl with homogenization buffer (4) and diluted with 100 μl of 2× reaction buffer (2.0 M diethanolamine [pH 8.0], 1.0 mM $MgCl_2$) to give a final volume of 200 μl. Then 20 μl of *p*-nitrophenyl phosphate (120 mM in 1× reaction buffer) was added to initiate the assay. The optical density at 405 nm was measured versus time. Controls included a blank with no cell lysate and cell lysate from uninfected cells. The protein content of each sample was determined by the method of Bradford (6), and AP activity was expressed as the rate of change in optical density divided by the protein concentration.

PCR. Vector DNA was quantitated by competitive amplification (17) by a modification of previously described methods (27). Total cellular DNA (125 ng) was amplified in a total reaction volume of 55 μl (0.01% gelatin, 50 mM KCl, 10 mM Tris [pH 8.5], 2 mM $MgCl_2$, 0.1 mM each of the four deoxynucleoside triphosphates, 0.1 mM [α-^{32}P]dTTP or 0.1 mM [α-^{32}P]dCTP, 1.5 U of *Taq* polymerase, and 125 ng of each *neo* primer, Neo1 and Neo5). In addition, 10 fg of plasmid pLASΔN (27) was added to all samples as a competitor for vector amplification. Amplification entailed 25 to 30 cycles of incubation at 95°C for 2 min followed by 72°C for 5 min. The reaction products were resolved in 5% nondenaturing acrylamide gels, which were then dried onto filter paper and exposed to film. Two PCR products were expected, a full-length *neo* band from the vectors LAPSN(PA317) and AAV-LAPSN and a shorter band, Δ*neo*, from pLASΔN. Signals from bands were quantitated by computer analysis of a digitized phosphorimage of the gel. The ratio of the full-length *neo* signal to the Δ*neo* signal was determined and compared with values obtained from reactions done with DNA samples containing known *neo* gene copy numbers per cell, made by mixing DNA from fibroblasts carrying a single integrated retroviral vector carrying *neo* with uninfected fibroblast DNA. This comparison allowed an estimation of the copy number of vector genomes per cell.

RESULTS

AAV vector transduction rates are reduced in primary versus immortalized airway epithelial cells. The performance of the AAV-LAPSN vector in primary airway epithelial cells was assessed by using epithelial cells obtained from nasal polyps of CF patients. Transduction efficiencies found in primary cells were compared with those obtained in an immortalized bronchial epithelial cell line, IB3, which has been used in previous studies as a target for transduction by AAV vectors (10–13).

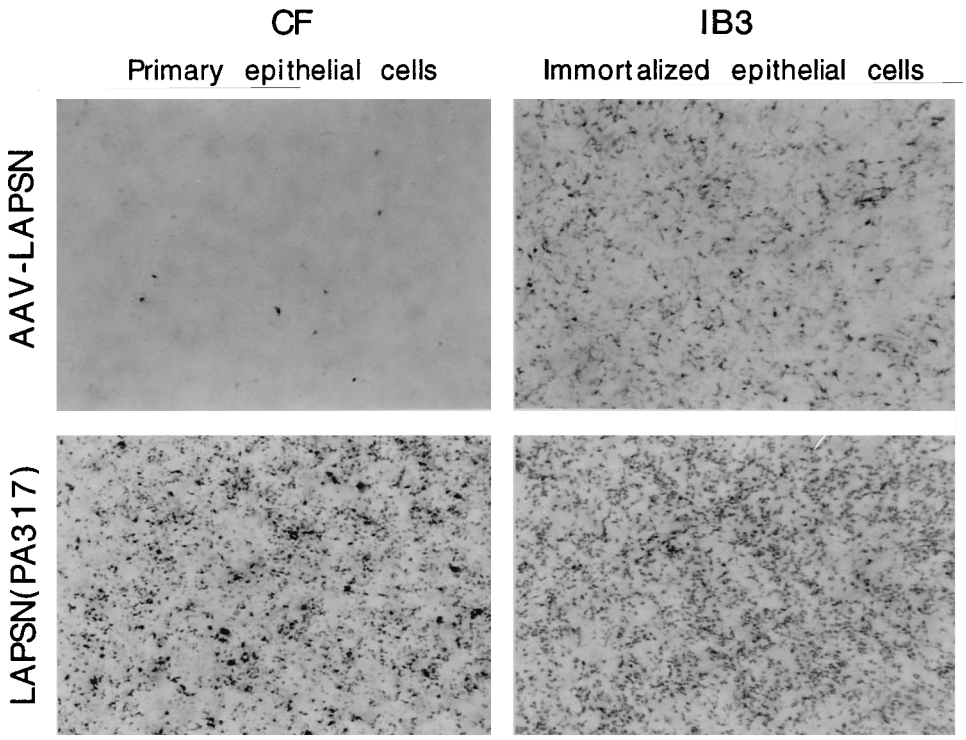

FIG. 2. AAV-LAPSN vector transduces primary human epithelial cells much less efficiently than immortalized human epithelial cells. Primary CF cells (left panels) and immortalized IB3 CF cells (right panels) were transduced by AAV-LAPSN (upper panels) or LAPSN(PA317) (lower panels) and stained for AP expression.

Cells were transduced by an overnight incubation with AAV-LAPSN. Transduced cells were stained for AP expression 2 days after infection, and the AP transduction rates were determined by counting stained foci of cells. In a parallel experiment, the retrovirus vector LAPSN(PA317) was used to provide a comparison of the efficiency of transduction in proliferating cultures of epithelial cells.

The AAV-LAPSN vector transduction rate was 60-fold higher in IB3 cells than in primary CF cells (Fig. 2, upper panels; Table 1). In contrast, the transduction rates of both cell types by the retroviral vector LAPSN(PA317) were equivalent (Fig. 2, lower panels; Table 1). Similarly, the AP activity in IB3 cells transduced by AAV-LAPSN was 50-fold higher than the AP activity in transduced primary CF cells (Table 1). At the multiplicity of infection used (0.1, based on the vector titer assayed on HeLa cells, or about 100 AAV vector particles per cell, based on DNA analysis of the virus stock), 0.1% of primary cells and 6% of IB3 cells were AP positive.

TABLE 1. AP transduction rates and activity in primary cells and cell lines[a]

Vector	Cells	Vector titer (FFU/ml)	Relative titer	Relative AP activity
AAV-LAPSN	CF	8×10^3	1	1
	IB3	5×10^5	60	50
LAPSN(PA317)	CF	5×10^5	1	ND[b]
	IB3	6×10^5	1	ND
AAV-LAPSN	HFF	1×10^3	1	1
	LNSV	1×10^4	10	40

[a] Monolayer cultures of cells were exposed to the AAV-LAPSN vector or the LAPSN(PA317) retroviral vector and stained for AP expression 2 days later. The results are given in AP focus-forming units per milliliter of virus. Nonconcentrated AAV vector stocks were used for fibroblast (HFF and LNSV) infections, while 30-fold-concentrated AAV vector stocks (generated by pelleting virions through 40% sucrose) were used for epithelial cell (CF and IB3) infections. Results are averages of at least two experiments except for the titer values for HFF and LNSV cells infected with AAV-LAPSN, which are an average of quadruplicate assays in a single experiment.

[b] ND, not done.

The rabbit has been used as a model for gene transfer by AAV vectors (11). We therefore examined the performance of AAV-LAPSN in primary rabbit tracheal epithelial cells and found that the transduction efficiency of the vector in rabbit cells was also low, similar to that found in primary CF nasal polyp cells (data not shown).

It was possible that the enhanced performance of AAV-LAPSN in the IB3 cells could have been due to an interaction of the SV40 regulatory sequence in the AAV-LAPSN vector with adenovirus or SV40 proteins expressed in the cell line, because the IB3 cell line was established by immortalization with an adenovirus-SV40 chimeric virus. To test this hypothesis, we used a vector that did not contain the SV40 sequence, AAV-Lβgeo. The transduction efficiency of AAV-Lβgeo was approximately 45 times better in IB3 cells than in two primary CF epithelial cell isolates (average of three independent experiments; data not shown). The relative transduction rates of AAV-Lβgeo in IB3 cells and CF cells paralleled those found with AAV-LAPSN, showing that the difference in the transduction efficiency of AAV vectors in primary and immortalized epithelial cells was independent of the SV40 promoter sequence and the marker genes used.

AAV vector transduction rates are reduced in primary versus immortalized human fibroblasts. To determine whether other cell types exhibited a similar phenomenon of low AAV

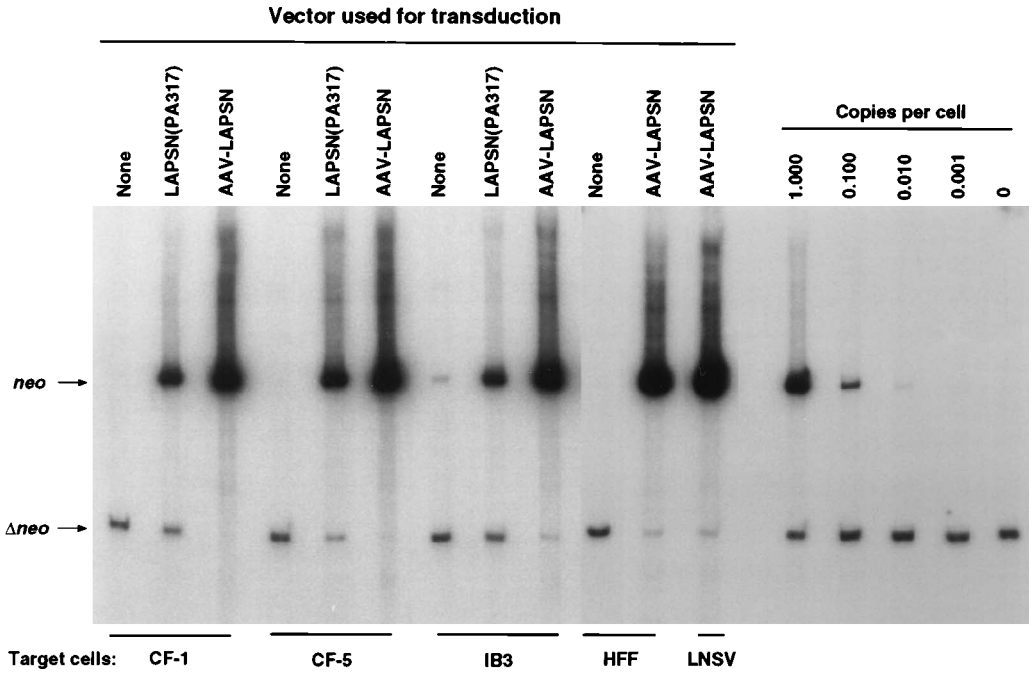

FIG. 3. Primary and immortalized cells are equally permissive for entry by AAV vector. Total cellular DNA from primary (CF-1 and CF-5) and immortalized (IB3) epithelial cells and DNA from nuclei of primary (HFF) and immortalized (LNSV) human fibroblasts was prepared 2 days after infection with retroviral or AAV vectors or after mock infection. Vector DNA in these samples was quantitated by competitive amplification as described in Materials and Methods. Amplification results from samples with known *neo* gene copy numbers are shown at right. The faint *neo* signal detected in the uninfected IB3 cells is most likely due to low-level contamination of this DNA preparation, as analyses of additional DNA samples from uninfected IB3 cells did not show a *neo* signal.

vector transduction rates in primary cells compared with that in immortalized cell lines, we examined AAV vector transduction in primary human fibroblasts and in the SV40-immortalized human fibroblast line LNSV (2). Transduction efficiency, measured by production of AP-positive cell foci, was 10-fold higher in LNSV cells than in primary fibroblasts (Table 1). Similarly, the AP activity in lysates of AAV-LAPSN-infected LNSV cells was 40-fold higher than that obtained in the primary human fibroblasts (Table 1). These results show that the efficiency of AAV vector transduction is reduced in primary fibroblasts compared with an immortalized fibroblast cell line, similar to the results obtained with primary and immortalized epithelial cells.

Lower transduction efficiency in primary cells is not due to reduced vector entry into cells. The low transduction efficiency of AAV vectors in primary cells could be explained by a reduced ability of the AAV vector to enter primary cells. To assess virus entry, we compared the relative amounts of vector genome in the cells by quantitative PCR. The PCR uses a known amount of a plasmid containing a deleted *neo* (Δ*neo*) gene to compete for primers to the *neo* gene. The copy number of the full-length *neo* gene in the cell resulting from vector delivery can be estimated from the ratio of the products derived from the full-length *neo* to those derived from the deleted *neo* gene. Two days after AAV or retroviral vector infection, epithelial cells were washed, trypsinized, and lysed to obtain total cellular DNA, while human fibroblasts were processed to obtain DNA from the nuclear fraction. In parallel, total cellular DNA was isolated from cells infected with the retrovirus vector LAPSN(PA317) at a low multiplicity of infection (0.1, based on titers assayed on HeLa cells). Copy number control DNAs were made by mixing DNA from a fibroblast cell line having a single integrated copy of a retrovirus carrying a *neo* gene with DNA from uninfected fibroblasts at appropriate ratios.

The autoradiograms in Fig. 3 show the results of the PCR experiment. The intensities of the *neo* bands were similar between primary and immortalized cells transduced by AAV-LAPSN, indicating that there were comparable amounts of amplifiable AAV vector DNA in primary and immortalized cells. Infection by LAPSN(PA317) also gave comparable amounts of amplified retrovirus vector DNA in primary and immortalized cell lines.

Quantitation of the PCR results by phosphorimage analysis was done to derive the *neo*/Δ*neo* ratio to estimate the number of *neo*-containing vector genomes in the cell. Primary epithelial cells and IB3 cells transduced by LAPSN(PA317) gave *neo*/Δ*neo* values of one to two copies of the LAPSN retroviral vector per cell. Multiple copies of the AAV-LAPSN vector genome were found in primary epithelial cells, IB3 cells, primary human fibroblasts, and LNSV cells. The *neo*/Δ*neo* ratios indicated that there were 20 to 40 copies of the vector genome per cell in all of these cell types. These results show that both immortalized and primary cells contained comparable numbers of AAV vector genomes. In addition, since nuclear DNA from human fibroblasts (human foreskin fibroblast) and LNSV cells) was analyzed in these experiments, we can conclude that a large amount of AAV vector DNA is present in cell nuclei and does not simply reflect virion binding to the cell surface. Therefore, differences in vector entry do not account for the differences seen in the efficiency of transduction by AAV vectors in primary and immortalized cells.

Southern analysis performed on extracted and undigested DNA resulted in detection of vector DNA in the low-molecular-weight region of the gel, and the intensity of the hybridizing signal was comparable between primary CF cells and IB3 cells

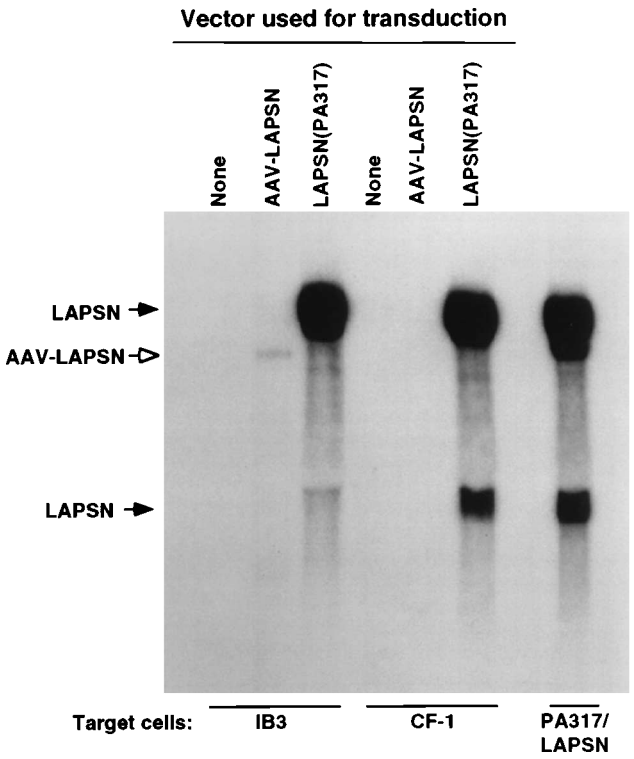

FIG. 4. Analysis of vector transcripts in human airway epithelial cells. Total cellular RNA harvested from cells 2 days after vector transduction or from PA317/LAPSN cells was analyzed by Northern (RNA) blotting with a *neo* probe. PA317/LAPSN cells are retrovirus packaging cells that produce the retrovirus vector LAPSN. The AAV vector-derived message (open arrow) and the retrovirus vector-derived messages (solid arrows) are indicated.

(data not shown). Vector DNA was not detected in the undigested genomic DNA located in the high-molecular-weight region of the gel. This result and similar results found with DNA extracted from the nucleus of primary human fibroblasts and LNSV cells are consistent with the hypothesis that the majority of AAV genomes remained episomal and not integrated into host chromosomes.

Level of AAV vector-derived mRNA in transduced populations of cells parallels AAV transduction efficiency. The AAV-derived mRNA in transduced IB3 cells was five times greater than background by phosphorimage analysis, while the mRNA in transduced CF cells was not detectable (Fig. 4). Together with the results above showing equivalent amounts of vector DNA in IB3 and CF cells, these results suggest that the block to functional transduction in primary cells occurs after virus entry but before vector transcription. Because we could not detect the vector mRNA in primary CF cells, we were unable to precisely quantitate the ratio of vector RNA in IB3 cells to that in CF cells, although we know it is greater than fivefold. Therefore, we cannot preclude the possibility that posttranscriptional events also contribute to the 60-fold difference in the observed transduction rates.

Although there were approximately 20 times more vector genomes in IB3 and CF cells transduced by the AAV-LAPSN vector than in those transduced by the LAPSN(PA317) vector (Fig. 3), the AAV-derived message was 100-fold less abundant by phosphorimage analysis than the retrovirus-derived message in IB3 cells and was not detectable in primary CF cells (Fig. 4, upper solid arrow). These results indicate that the majority of AAV vector genomes in IB3 and primary CF cells were not transcriptionally active.

Events secondary to the presence of immortalizing genes enhance transduction by AAV-LAPSN. To determine whether the presence of oncogenes or immortalizing genes was sufficient to enhance transduction by AAV vectors, primary CF cells were genetically modified with the retrovirus vector LXSN16E6E7, which contains the E6 and E7 immortalizing genes of HPV type 16. LXSN16E6E7 has been shown to efficiently immortalize primary human foreskin epithelial cells (15). Primary human foreskin fibroblasts were also transduced with the same vector or another carrying the SV40 large T antigen (Ziptex) (7). Within one to five passages after drug selection, cells were exposed to AAV-LAPSN, and the relative AP activity was determined 48 h following infection.

Nasal polyp cells containing the HPV-immortalizing genes and human foreskin fibroblasts containing either the HPV-immortalizing genes or the SV40 T antigen gene did not exhibit an increase in relative AP activity over control cells (Fig. 5A). Therefore, the presence of immortalizing genes from

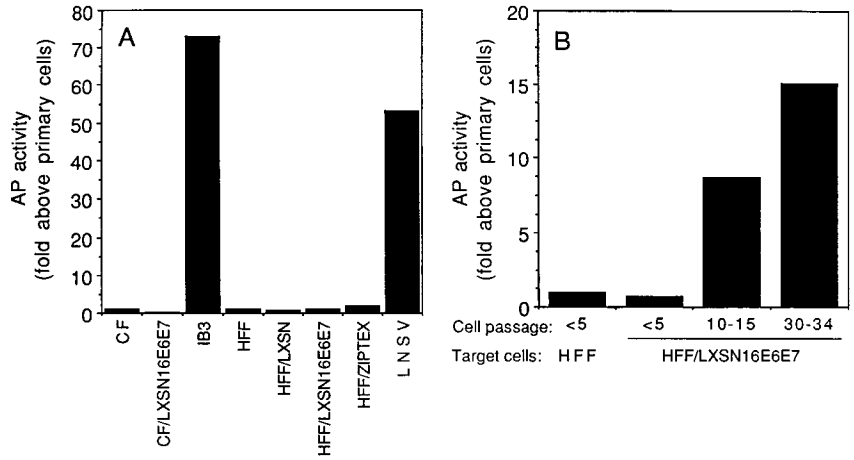

FIG. 5. Cells harboring immortalizing genes acquire a permissive phenotype for AAV transduction after long-term culture. The relative AP activity in cell lysates 2 days after AAV-LAPSN transduction is shown. (A) AP activities are shown for primary (CF and HFF) and immortalized (IB3 and LNSV) human cells and cells containing the immortalizing genes of HPV type 16 (CF/LXSN16E6E7 and HFF/LXSN16E6E7), the SV40 T antigen (HFF/Ziptex), or a control retroviral vector carrying only the *neo* gene (HFF/LXSN). (B) Effect of passage history on AP activity in immortalized (HFF/LXSN16E6E7) cells after AAV-LAPSN infection.

HPV or SV40 was not sufficient for the enhancement of transduction by the AAV vector. Additionally, the fact that the Ziptex vector did not enhance AAV transduction is in agreement with our previous conclusion that the enhanced transduction efficiency seen in IB3 cells did not involve interaction of the SV40 T antigen with vector regulatory sequences.

During the establishment of an immortalized cell line, the population of cells often experience cell crisis, which is reflected by poor plating efficiency, slow growth, and senescence of a portion of the population. Various cellular changes due to genetic instability are likely to occur during this stage. The IB3 and LNSV cell lines have undergone many passages in tissue culture and thus have probably accumulated these changes, whereas the populations that we analyzed immediately after introduction of immortalizing genes have not. We therefore examined the ability of AAV-LAPSN to transduce cultures of cells at middle and late passages.

Human foreskin fibroblasts containing HPV immortalizing genes transduced by AAV-LAPSN at early passage (less than five passages) showed no difference in AP activity from similarly transduced primary cells. At middle passage (10 to 15 passages), their AP activity following AAV-LAPSN transduction was eight- to nine-fold greater than that of the primary cells, and at late passage (30 to 34 passages), their AP activity was 15 times greater than in the primary cells (Fig. 5B). These results show that in long-term culture, changes occur that increase the permissiveness of cells for transduction by AAV vectors.

DISCUSSION

We have shown that AAV vectors transduce primary human cells markedly less well than they transduce immortalized cells. In our investigation into the basis for the enhanced expression in immortalized cells, we first focused on the interaction of possible transactivating factors with vector sequences. The enhanced transduction of AAV-LAPSN in the IB3 cell line was not due to interaction of adenovirus or SV40 transactivating proteins particular to this cell line with the SV40 promoter in AAV-LAPSN, because a vector (AAV-Lβgeo) that did not contain this sequence also demonstrated enhanced transduction of IB3 cells. Also, introduction of the SV40 large T antigen into primary human fibroblasts did not result in a greater transduction efficiency, indicating that enhanced transduction by AAV vectors in LNSV was not mediated directly through the SV40 large T antigen present in this cell line. Similarly, introduction of immortalizing genes derived from HPV type 16 did not result in a greater permissiveness for transduction by AAV vectors in either primary human epithelial cells or primary human fibroblasts. The fact that the retrovirus vector LAPSN(PA317), which contains an AP expression cassette that is similar to that of AAV-LAPSN, did not exhibit a difference in its ability to transduce primary and immortalized cell line suggests that the differences observed with AAV vectors are intrinsic to the biology of AAV vectors and not a function of the expression cassette used.

Primary and immortalized cells were equally susceptible to AAV vector entry, because the PCR analysis indicated similar amounts of vector sequences in these cells. The copy number of 20 to 40 genomes per cell found 2 days postinfection is consistent with our estimate of a multiplicity of infection of 0.1 (based on vector titer assayed on HeLa cells), or approximately 100 particles per cell. Thus, AAV vectors exhibit efficient entry of vector genomes into cells. However, the vast majority of vector genomes remained episomal and unintegrated. In addition, we know that the episomal DNA is predominantly single-stranded (1, 30). We were not able to estimate the amount of vector genome that was integrated either because the signal was below the limit of detection by Southern analysis or because the large amount of unintegrated AAV vector DNA may have obscured the signal.

Enhanced transduction appears to occur at a step prior to translation, because a difference in the abundance of AAV vector-derived message between primary and immortalized cells was detected. Processes occurring after virus entry and before message translation involve second-strand synthesis, integration of vector genome, and transcription from the integrated provirus or possibly from the episomal vector. The change in permissiveness found in late-passage immortalized cells may involve one or more of these steps.

Introduction of the immortalizing genes of HPV type 16 or SV40 into primary cells did not make the cells more susceptible to transduction by AAV-LAPSN. HPV type 16 E6 and E7 proteins bind p53 and the retinoblastoma gene product (Rb), respectively (9, 33), and the SV40 T antigen binds both proteins (8, 28). Therefore, the binding of these regulatory proteins is not sufficient for the permissive phenotype. However, inactivation of Rb and p53 can lead to a loss of cell cycle control (19, 23) and the accumulation of further mutations as a consequence. p53 has been shown to arrest cells that accumulate DNA damage (21). The inactivation of this regulatory protein may allow the accumulation of additional mutations that resulted in the acquisition of the permissive phenotype. This might explain why late-passage cells containing the immortalizing genes of HPV type 16 are more permissive for transduction by an AAV vector.

An AAV vector that transfers the *neo* gene has been shown to transduce the IB3 cell line with an efficiency of 60 to 70% at a particle-to-cell ratio of approximately 800 to 1,000 (13). Our experiments with AAV-LAPSN in an IB3 cell line yielded a 6% transduction efficiency at a particle-to-cell ratio of approximately 100, which is comparable to the results referenced above after accounting for the difference in the multiplicity of infection. However, the transduction efficiency of the AAV vector in primary airway epithelial cells was only 0.1% at a particle-to-cell ratio of 100, dramatically lower than in immortalized cells. It has been shown that functional correction of the CF chloride ion channel defect requires that approximately 6 to 10% of the cells be genetically modified (18). Our results from analysis of primary airway epithelial cells suggest that a particle-to-cell ratio of 6,000 may be required for AAV vectors to achieve the 6% transduction efficiency required for effective CF gene therapy. Additionally, our study focused on analysis of actively dividing cells, which have been shown to be preferentially transduced by AAV vector by 200-fold over stationary cells (30). The particle-to-cell ratio required in the airway epithelium, where the proliferation rate is low, might then be about 10^6. In fact, the actual value may be an order of magnitude smaller because of the ability of AAV vector episomal DNA to be recruited for transduction as cells divide (1, 30). Given that maximum AAV vector particle number in highly purified stocks is at most 10^{10} particles per ml, and given the large number of epithelial cells in the airway, our calculations predict that it will be difficult to deliver enough virus to treat CF airway disease.

The results of the experiments in primary and immortalized cells clearly demonstrate that the intracellular environment greatly affects the transduction efficiency of AAV vectors. Therefore, it is possible that the transduction efficiency of AAV vectors in primary cells undergoing differentiation in vivo is substantially different from that which occurs in a proliferative monolayer in vitro. In vivo delivery of an AAV vector into

the rabbit airway has been demonstrated by using a vector carrying the human CFTR cDNA (11). AAV vector sequences were detected by in situ PCR. However, our study shows that PCR analysis for vector genomes is not a valid measurement of transduction efficiency, because the majority of AAV genomes appear to be transcriptionally inactive. Although detection of human CFTR protein in situ was possible (11), quantitation of the efficiency of transduction by protein staining has not been reported.

We have compared the transduction efficiency of two viral vector systems in primary and immortalized cells. The transduction by AAV vectors was dramatically less efficient in primary cells than in immortalized cells, while there was little difference in the performance of the retrovirus vector in the same cells. Thus, the behavior of AAV is strongly affected by the host cell environment. It is likely that in the absence of helper virus, AAV is dependent on host factors for efficient second-strand synthesis, integration, and expression. These cellular factors may be limiting in primary cells and altered in immortalized cells. In conclusion, the results of our study show that AAV infection of immortalized cells does not accurately reflect the situation in primary cells, and it will therefore be important to evaluate primary cells as models for studies on AAV biology and AAV vector-mediated gene therapy.

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