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Adeno-associated virus vectors preferentially transduce cells in S phase

(gene therapy/cell cycle)

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ABSTRACT **Vectors based on adeno-associated virus can stably transfer genes by chromosomal integration in recipient cells. In this study we have infected stationary and dividing primary human fibroblast cultures with adeno-associated virus vectors encoding alkaline phosphatase and neomycin phosphotransferase. We find that the transduction frequency of S phase cells is about 200 times that of non-S phase cells. However, neither S phase nor mitosis is essential for transduction. Single-stranded vector genomes survive in stationary cultures and can be recruited for transduction by stimulating these cultures to divide. Stable transductants contain randomly integrated vector sequences. These findings have important implications for the use of adeno-associated virus vectors in gene therapy.**

Adeno-associated virus (AAV) is a dependent human parvovirus that usually requires the presence of a helper virus such as adenovirus or herpes virus for productive viral infection (1, 2). AAV is capable of integration into the host chromosome in the absence of helper virus and often integrates in the same location on chromosome 19 (3–5). By expressing essential viral genes in trans, several investigators have developed replication-incompetent, integrating, transducing vectors based on AAV (6–9). These vectors represent an alternative to the retroviral vectors currently used in many gene therapy protocols (10, 11). Retroviral vectors based on murine leukemia virus (MLV) require cell division for efficient transduction (12), and this limits their usefulness in clinical applications. We therefore set out to determine if transduction by vectors based on AAV requires cell division.

Little is known about the relationship between the cell cycle and infection by dependent parvoviruses such as AAV. Replication of the related autonomous parvoviruses has been shown to occur in S phase (1, 13). This may be due to a requirement for host DNA polymerases in viral replication, as autonomous parvoviruses do not encode a polymerase, and cellular DNA polymerase inhibitors decrease their replication *in vitro* (14–16) and *in vivo* (17). AAV is capable of replicating at a reduced level without helper virus when synchronized cultures are enriched for S phase cells (18), suggesting similarities to the autonomous parvoviruses. Although transduction by AAV vectors does not require viral replication as in a productive infection, vector gene expression and possibly integration presumably require conversion of single-stranded input genomes to double-stranded molecules by host polymerases, and this may occur preferentially in S phase.

In the experiments presented here we have examined the transduction of normal human fibroblasts by AAV vectors in stationary and dividing cultures. We find that vector genomes can persist in stationary phase cells, but transduction preferentially occurs in cells that have entered S phase.

MATERIALS AND METHODS

Cell Culture. 293 cells, PA317 packaging cells, and the primary human foreskin fibroblasts used have been described (19–21). Except for stationary cultures, cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS) at 37°C in 10% CO_2. Primary human fibroblasts were obtained from frozen stocks (passage 3 or 4) and maintained in culture for <10 additional passages. Stationary cultures were prepared by changing the medium in confluent cultures to DMEM containing 5% heat-inactivated FBS and 1 μM dexamethasone and maintaining these cultures for at least 2 weeks while replacing the medium every 3–4 days. Dividing cultures were prepared by treatment with trypsin and plating the cells at a density of either 2.5×10^5 cells per 35-mm well in six-well plates (Falcon) or 4×10^5 cells per 60-mm dish (Corning) the day before infection.

Vector Construction and Production. The plasmids pTR, pTRneo, and pTRAAVneo were kindly provided by Sergei Zolotukhin and Nicholas Muzyczka (State University of New York, Stony Brook) and were derived from the vector plasmid dl3-94 (8). pTR and pTRneo are vector cloning constructs containing no insert or the simian virus 40 early promoter and neomycin phosphotransferase gene (*neo*), respectively. pTRAAVneo is a helper construct containing the AAV terminal repeats, AAV coding region, and the *neo* gene. pALAPSN was constructed from the pTR backbone (see Fig. 1; sequence available on request). Plasmids pTRneo and pALAPSN were used to generate vector stocks of AAV-SVNEO and AAV-LAPSN, respectively, as described (ref. 6; see Fig. 1). When necessary, vector stocks were concentrated as described (22). Based on the infectious center assay (18), this method produced stocks with wild-type virus titers at ≈2% of vector titers (data not shown). AAV vector particle numbers were determined by quantitation of purified vector DNA on Southern blots probed with vector sequences. The retroviral vector PA317/LAPSN (23) is structurally analogous to AAV-LAPSN.

Transduction Assays. Transduction of G418 resistance was performed by adding vector dilutions to cultures on day 1, treating with trypsin and plating the cells at different dilutions in DMEM with 10% heat-inactivated FBS on day 2, and adding G418 to a concentration of 600 μg/ml active drug on day 3. Infections with retroviral vectors were performed in the presence of 4 μg of Polybrene per ml (Sigma). Cultures were maintained in medium containing G418 for ≈2 weeks with medium changes every 3–4 days until uninfected control

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Abbreviations: AAV, adeno-associated virus; FBS, fetal bovine serum; LTR, long terminal repeat; MLV, murine leukemia virus.

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dishes contained no living cells; then surviving colonies were counted after staining with Coomassie brilliant blue G. Primary fibroblasts transduced by AAV vectors often formed small colonies, and sometimes only individual cells survived selection. AAV vector sequences were present in five of five such individual surviving cells isolated and subsequently grown without G418 selection (data not shown), confirming that they are true transductants. Transduction of alkaline phosphatase was measured by staining cells 2 days after infecting cultures and counting individual transduction events (24).

DNA Manipulations. Restriction digests, plasmid manipulations, genomic DNA isolation, and Southern blots were performed using standard procedures (25). AAV vector DNA was purified by the procedure of Samulski *et al.* (9). Episomal and high molecular weight DNAs were fractionated by the Hirt procedure (26), followed by proteinase K digestion, extraction with phenol, chloroform, and butanol, and ethanol precipitation. Subcellular fractionation experiments showed that the majority of input AAV vector genomes are inside the nucleus at 2 days after infection (data not shown).

Autoradiography. Cultures were labeled with 10 μCi of [^{3}H]thymidine per ml (DuPont/NEN; 89 Ci/mmol; 1 Ci = 37 GBq) using dialyzed, heat-inactivated FBS. After labeling, cells were either stained or washed and cultured in the presence of 20 μM nonradioactive thymidine until staining. Cultures were first stained for alkaline phosphatase (24), rinsed with distilled water, treated with cold 5% trichloroacetic acid, and processed for autoradiography as described (12); they were then counterstained with nuclear fast red (1 mg/ml in 5% aluminum sulfate) to identify unlabeled nuclei.

RESULTS

Transduction of Fibroblasts by AAV Vectors. We chose contact-inhibited, primary human fibroblasts as a model system to study transduction of nondividing cells. When confluent fibroblast monolayers are maintained in 5% FBS and 1 μM dexamethasone for 2 weeks, 4% of these cells pass through S phase in 24 hours as determined by [^{3}H]thymidine incorporation (data not shown). The remainder of the cells in these stationary cultures represent a non-dividing, normal cell population. In growing cultures plated in 10% FBS, ≈60% of cells pass through S phase in 24 hours (data not shown). If transduction only occurs in dividing cells, then we would expect transduction frequencies to be 15 times higher in dividing cultures than stationary cultures (60% dividing/4% dividing).

Table 1 shows the titers of different viral vectors on stationary and dividing fibroblast cultures. Cells were infected with the AAV-based vector AAV-SVNEO (Fig. 1), treated with trypsin, plated at different dilutions, and then grown in the presence of G418 (see *Materials and Methods*). In three separate experiments, the number of G418-resistant colonies was similar after infection of stationary and dividing cultures (S/D ratio; Table 1). As a control, the same experiment was performed with the analogous amphotropic retroviral vector PA317/LAPSN, which also contains the *neo* gene transcribed from the simian virus 40 early promoter (23). The PA317/LAPSN titer was consistently 10- to 20-fold lower after infecting stationary cultures as compared to dividing cultures, in agreement with previous experiments demonstrating that these retroviral vectors require cell division for efficient transduction (12).

Table 1. Vector titers on stationary and dividing fibroblast cultures

Viral vector	Titering method	Titer, units/ml		S/D ratio*
		Stationary	Dividing	
AAV-SVNEO	G418 resistance	2.8×10^5	4.3×10^5	0.65
		1.0×10^5	4.8×10^4	2.1
		6.0×10^4	1.3×10^5	0.46
				1.07†
PA317/LAPSN	G418 resistance	5.0×10^4	1.1×10^6	0.045
		4.3×10^4	3.8×10^5	0.11
		3.0×10^4	7.1×10^5	0.042
				0.066†
AAV-LAPSN	Alkaline phosphatase	3.1×10^2	1.5×10^4	0.021
		8.0×10^2	2.1×10^4	0.038
				0.030†

*Stationary titer/dividing titer.
†Average ratio.

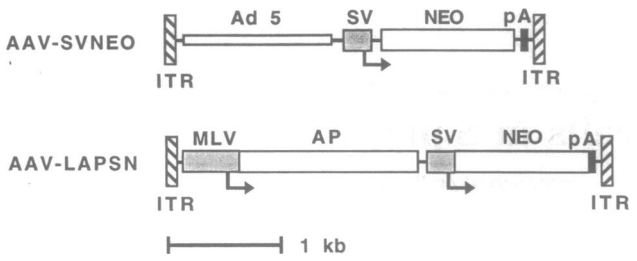

FIG. 1. Maps of the AAV vectors AAV-SVNEO and AAV-LAPSN. The positions of the AAV inverted terminal repeats (ITR), simian virus 40 early (SV) and MLV long terminal repeat (LTR) (MLV) promoters, human placental alkaline phosphatase (AP) and neomycin phosphotransferase (NEO) genes, and simian virus 40 poly(A) signal (pA) are shown. Arrows indicate transcriptional start sites. AAV-SVNEO contains the adenovirus 5 *Bgl* II J fragment (Ad 5) to increase vector size.

These results suggest that AAV vectors differ from retroviral vectors in their ability to transduce nondividing cells. However, the assay for G418 resistance requires that stationary cultures be treated with trypsin after vector exposure and undergo cell division during the selection period. Thus it is possible that transduction of fibroblasts by AAV vectors only occurs after stationary cultures are stimulated to divide. To address this possibility, we used an AAV vector containing the alkaline phosphatase gene under the control of the MLV LTR promoter (AAV-LAPSN; see Fig. 1). Transduction of the alkaline phosphatase gene can be monitored by staining 2 days after infection and does not require that stationary cultures be allowed to divide. When the alkaline phosphatase titers of AAV-LAPSN were measured, the titer on dividing cultures was >20 times that on stationary cultures (Table 1). This is in contrast to the nearly equal G418 resistance titers of AAV-SVNEO and suggests that the majority of cells in stationary fibroblast cultures are in fact resistant to transduction by AAV vectors unless they are subsequently stimulated to divide.

Alkaline Phosphatase Transduction and [^{3}H]Thymidine Incorporation. To determine which cells in a stationary culture can be transduced by AAV vectors, we performed an experiment that measured on a single cell level both transduction and passage through S phase. Stationary fibroblast cultures were infected with AAV-LAPSN in the presence of [^{3}H]thymidine and then stained for alkaline phosphatase expression and subjected to autoradiography. In this assay, cells expressing alkaline phosphatase have a dark cytoplasm, and cells passing through S phase have dark autoradiography grains over the nucleus. Fig. 2 *A–C* show examples of transduced cells that had passed through S phase, and Fig. 2*D* shows a transduced cell with an unlabeled nucleus. As summarized in Table 2, ≈90% of cells expressing alkaline phosphatase have incorporated [^{3}H]thymidine, whether or not the labeling period included the days before and after infection. Given that 4% of all cells in the culture pass through

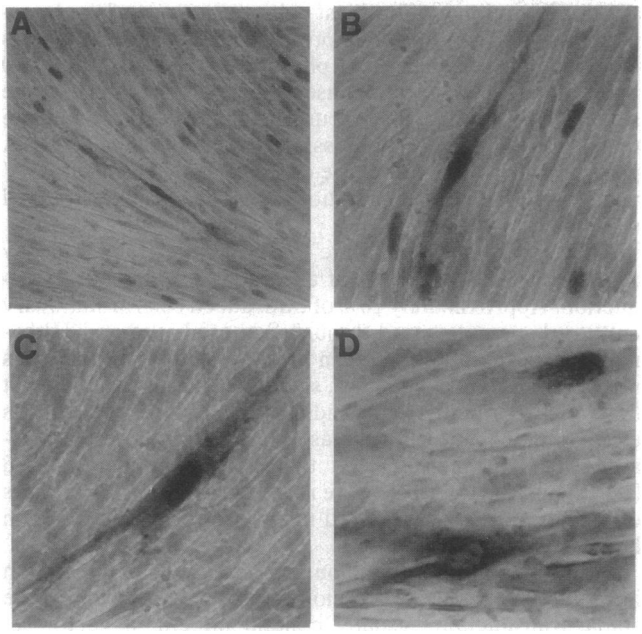

FIG. 2. Transduction of alkaline phosphatase by AAV-LAPSN and thymidine incorporation. Stationary fibroblast cultures infected with AAV-LAPSN in the presence of [^{3}H]thymidine were stained for alkaline phosphatase expression (darkly stained cytoplasm), covered with nuclear emulsion, and exposed for autoradiography (S phase cells have nuclear autoradiography grains); they were then counterstained with nuclear fast red. Photomicrographs of cells expressing alkaline phosphatase are shown with labeled (*A–C*) or unlabeled (*D*) nuclei. *A* is at a lower magnification.

S phase in 24 hr, the relative transduction rates of S phase (90%/4%) and non-S phase (10%/96%) cells can be calculated. Based on these numbers, S phase cells are transduced at ≈200 times the frequency of non-S phase cells [(90/4)/(10/96) = 216], and we can conclude that AAV-LAPSN preferentially transduces the small proportion of S phase cells present in stationary cultures.

Episomal AAV Vector Genomes Persist in Stationary Phase Cells. The different AAV vector titers on stationary cultures as measured by G418 resistance and alkaline phosphatase expression (Table 1) can be explained if entering vector genomes survive in stationary phase cells, but expression of the transferred gene occurs after these cells enter S phase. We therefore performed experiments to determine the status of AAV vector genomes in stationary cultures. Episomal DNA was isolated by the method of Hirt (26) from stationary fibroblast cultures up to 12 days after being infected with AAV-SVNEO at a high multiplicity (470 vector particles per cell). Fig. 3*A* shows a Southern blot of these Hirt supernatant DNAs probed for *neo* sequences, and Fig. 3*B* plots the amount of single-stranded vector genomes present over time. Although the number of vector genomes decreases with time, even at 12 days there are 45 vector genomes present per cell, or ≈1/10th of the inoculum. Southern blots performed with high molecular weight genomic DNA from Hirt pellets of the same samples showed only contaminating episomal vector sequences (data not shown). Thus the majority of AAV-SVNEO sequences present in stationary cultures is in the form of single-stranded genomic molecules, and these presumably represent surviving input genomes.

Table 2. [^{3}H]Thymidine incorporation in fibroblasts transduced by AAV-LAPSN

Labeling period			Transduced AP+ cells		
Day −1	Day 0	Day 1	Total no.	No. ^{3}H+	% ^{3}H+
−	+	−	170	150	88
+	+	−	265	238	90
−	+	+	124	112	90
+	+	+	546	501	92

Autoradiography and staining for alkaline phosphatase expression were performed on stationary fibroblast cultures after infection with AAV-LAPSN. Infection was on day 0. [^{3}H]Thymidine was present on the indicated days. Cells staining positive for alkaline phosphatase (AP+) and showing nuclear [^{3}H]thymidine uptake (^{3}H+) were determined by microscopy as in Fig. 2. Each result is the total from at least two independent experiments.

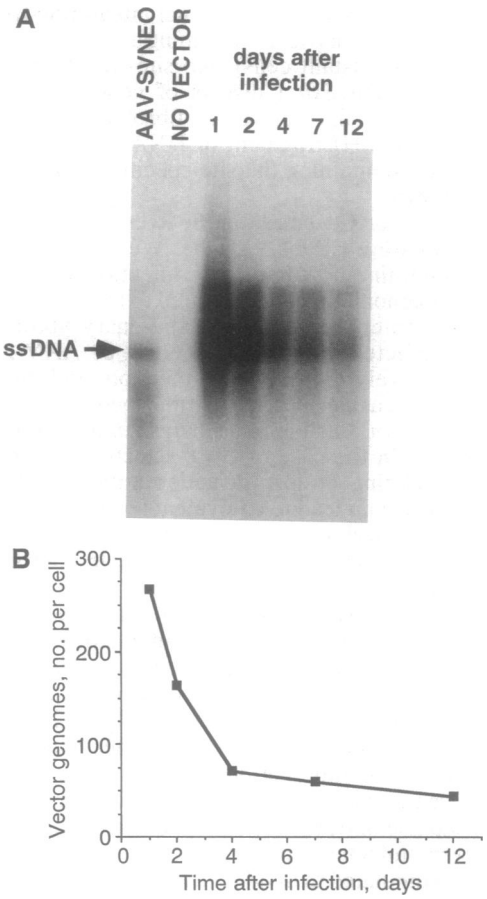

FIG. 3. Persistence of AAV-SVNEO in stationary fibroblast cultures. Hirt supernatants were isolated from stationary fibroblast cultures at different times after infection with AAV-SVNEO (470 particles per cell). (*A*) Southern blot of these DNA samples probed with *neo* sequences. The AAV-SVNEO lane contains vector DNA purified from particles. The NO VECTOR lane contains DNA from an uninfected culture. The position of single-stranded, monomer vector genomes is indicated (ssDNA). (*B*) ssDNA bands from *A* were quantified on a PhosphorImager 400E (Molecular Dynamics) and the results are plotted as vector genomes per cell.

AAV Vector Integration Is Inefficient and Random. Although vector integration was not detected in the experiment described above, integration does occur in the cells that eventually form G418-resistant colonies. Southern blot analysis of 17 independent, G418-resistant fibroblast clones transduced by AAV-LAPSN at a multiplicity of 700 particles per cell confirmed the presence of single, randomly integrated proviruses in 15 clones, and two or three integrated proviruses in 2 clones (based on the random sizes of vector restriction fragments obtained after digestion outside of vector sequences; data not shown). There was no evidence for episomal vector sequences. Thus, about 1 in 600 infecting vector particles successfully establishes an integrated provirus in G418-resistant cells. As only 17% of cells were trans-

duced in this experiment, the minimal integration frequency was 1 in 3500 vector particles (assuming all integration events produced G418-resistant cells). No site-specific integration was detected when *Ssp* I digests of genomic DNA from transduced clones and uninfected fibroblasts were probed with a 2.6-kb *Bam*HI fragment of DNA from human chromosome 19 (5) containing the site-specific integration locus (data not shown).

Surviving Vector Genomes Can Be Recruited for Transduction by Stimulating Cells To Divide. We performed experiments to determine how long after infection surviving, episomal vector genomes can be recruited to transduce cells that subsequently enter the cell cycle. Stationary fibroblast cultures were infected with AAV-SVNEO or AAV-LAPSN, washed extensively the day after infection, and then left in culture for increasing lengths of time before assaying for G418 resistance or alkaline phosphatase expression, respectively (Fig. 4). In the case of G418 selection, trypsin treatment and replating at low densities stimulate the entire culture to divide and lead to equivalent recruitment of vector genomes for transduction at all time points, with titers comparable to those on dividing cultures. In contrast, alkaline phosphatase transduction occurs in a culture with 4% of the cell population passing through S phase each day, producing increasing recruitment over time and titers that rise >30-fold after 12 days to a level comparable to dividing culture titers. These results suggest that vector genomes can be recruited for transduction for at least 12 days after infection. As the number of vector genomes present in cells decreases over this time period (Fig. 3), the surviving population of vector molecules may be enriched in competent genomes capable of transduction. When stationary fibroblast cultures were infected with ≈5000 AAV-LAPSN particles per cell and stimulated to divide the next day, 28% of the cells were resistant to G418 (data not shown). Thus a significant percentage of cells in stationary fibroblast cultures can recruit AAV vector genomes for transduction, although high infection multiplicities are required.

DISCUSSION

Cell Cycle Effects on Transduction by AAV Vectors. We have shown that the majority of cells transduced by AAV vectors in stationary fibroblast cultures are part of a subpopulation of proliferating cells present in these cultures. Cells that have passed through S phase are transduced at >200 times the rate of non-S phase cells. While there may be several cellular processes occurring in S phase that stimulate transduction, one likely possibility is that cellular DNA polymerases active in S phase convert single-stranded AAV vector genomes to transcriptionally active double-stranded molecules.

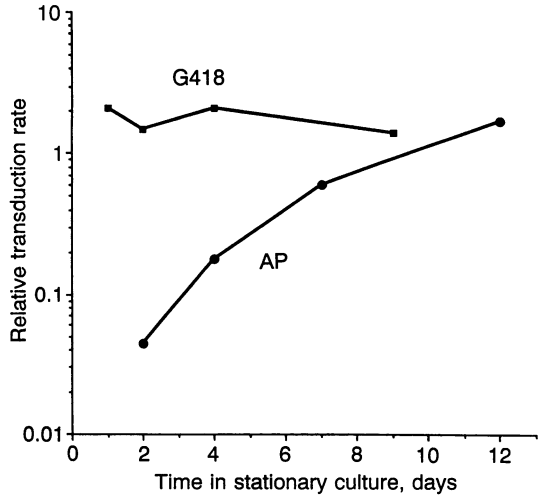

FIG. 4. Recruitment of vector genomes for transduction over time. Stationary human fibroblast cultures infected with AAV-SVNEO or AAV-LAPSN were assayed for G418 resistance (G418) or alkaline phosphatase expression (AP), respectively, at the indicated time after infection (days in stationary culture). The relative transduction rate is the rate observed in stationary culture at the indicated time divided by the rate observed in dividing cultures (see text).

Although S phase cells are preferentially transduced by AAV vectors, S phase is not absolutely required for transduction. Approximately 10% of transduced cells in stationary cultures have not passed through S phase based on [^{3}H]thymidine incorporation. We can also conclude that mitosis is not required for transduction, since the transduced cells that have incorporated [^{3}H]thymidine are isolated, single cells expressing alkaline phosphatase and are only rarely associated with neighboring cells that have also incorporated [^{3}H]thymidine. If mitosis were required, there would be pairs of daughter cells with labeled nuclei wherever transduction had occurred.

Transduction and Integration. It is not known if integration is required for gene expression from AAV vectors. While stable transductants usually contain integrated vector sequences (our results; refs. 6–9 and 27), double-stranded, episomal vector sequences have also been described (8). Episomes could be generated by recombination and excision of integrated vector sequences, or they may replicate autonomously, as the AAV terminal repeat can function as an origin in the absence of viral gene products (28). Transient gene expression could also occur from input AAV vector genomes without integration, if they are converted to double-stranded molecules by DNA synthesis or base-pairing with complementary input molecules.

Our results suggest that most transduction events are associated with vector integration. If transient gene expression occurred from episomal vector sequences, the alkaline phosphatase titers of AAV-LAPSN should be higher on dividing cells than the G418-resistance titers, as the former method does not require stable gene expression. However, the titers are similar using both methods (unpublished observations), and all G418-resistant transductants analyzed to date contained integrated vector sequences. In addition, the low integration frequency of 1/600–1/3500 per vector genome in primary fibroblasts correlates with the low transduction efficiency per particle of AAV vectors, suggesting that integration is the rate-limiting step in transduction. We have found that 700–5000 particles per cell are required to transduce 17–28% of primary fibroblasts, and similar results have been obtained with transformed cell lines, which require 850–1000 vector particles per cell to transduce 3–70% of cells (6, 9, 29). The integration frequency of wild-type AAV is of the same magnitude, as infection multiplicities of 200–500 virus particles per cell are required to latently infect 2–30% of a cultured cell population (30–32).

Although the frequent site-specific integration of wild-type AAV proviruses in chromosome 19 is well documented (3–5), all primary fibroblast transductants we analyzed had no rearrangements in the site-specific integration locus. Thus AAV vectors integrate randomly in primary fibroblasts. As suggested previously (27, 33), additional viral sequences not present in many vectors may be required for efficient site-specific integration. It is possible that site-specific integration leads to transcriptional repression that would normally facilitate viral latency but also prevents transduction by AAV vectors and results in a selection bias for random integration events. However, if silent site-specific vector integration occurs it must be infrequent, since secondary site-specific integration events were not observed in transduced fibroblasts infected at high multiplicities.

Implications for Gene Therapy. One disadvantage of AAV vectors is their inefficient integration per vector particle. Although a significant percentage of cultured cells can be transduced *in vitro* at high multiplicities of infection, it is not clear how effective this will be *in vivo*. Thousands of vector particles are required to transduce a single normal, dividing fibroblast, in a culture system lacking immune mediators, mucous secretions, digestive enzymes, and other potential inhibitors of transduction likely to be encountered *in vivo*. The large numbers of vector particles required to transduce a significant percentage of cells in a human organ may limit the effectiveness of AAV vectors in clinical situations.

The ideal vector for many gene therapy applications would be capable of efficient and stable integration in nondividing cells such as hematopoietic stem cells, quiescent lymphocytes, and normal epithelial cells. The retroviral vectors based on MLV that are currently used require dividing cells for efficient transduction (12), apparently because viral particles do not enter the nucleus until mitosis (34). AAV vectors also preferentially transduce dividing cells, but it is S phase and not mitosis that stimulates transduction. If cellular DNA polymerases are required, there may be ways to increase transduction by inducing unscheduled DNA synthesis in nondividing cells. The finding that transduction occurs at a reduced frequency in non-S phase cells suggests that this is possible.

Because AAV vectors persist in stationary cultures, and can be recruited for transduction when cells are stimulated to divide, there may be a long window of opportunity for transduction. Vector genomes survive at least 12 days with no apparent loss in transducing ability and may survive much longer, as the rate of genome loss from stationary cultures decreases with time. This could allow for transduction of slowly dividing cell populations and may be particularly important for hematopoietic stem cells and continuously repopulating epithelial cells such as those in the respiratory and gastrointestinal systems.

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