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# Efficient Gene Transfer into Nondividing Cells by Adeno-Associated Virus-Based Vectors

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**Gene transfer vectors based on adeno-associated virus (AAV) are emerging as highly promising for use in human gene therapy by virtue of their characteristics of wide host range, high transduction efficiencies, and lack of cytopathogenicity. To better define the biology of AAV-mediated gene transfer, we tested the ability of an AAV vector to efficiently introduce transgenes into nonproliferating cell populations. Cells were induced into a nonproliferative state by treatment with the DNA synthesis inhibitors fluorodeoxyuridine and aphidicolin or by contact inhibition induced by confluence and serum starvation. Cells in logarithmic growth or DNA synthesis arrest were transduced with vCWR:βgal, an AAV-based vector encoding β-galactosidase under Rous sarcoma virus long terminal repeat promoter control. Under each condition tested, vCWR:βGal expression in nondividing cells was at least equivalent to that in actively proliferating cells, suggesting that mechanisms for virus attachment, nuclear transport, virion uncoating, and perhaps some limited second-strand synthesis of AAV vectors were present in nondividing cells. Southern hybridization analysis of vector sequences from cells transduced while in DNA synthetic arrest and expanded after release of the block confirmed ultimate integration of the vector genome into cellular chromosomal DNA. These findings may provide the basis for the use of AAV-based vectors for gene transfer into quiescent cell populations such as totipotent hematopoietic stem cells.**

Rapid progress in molecular biology has revealed defined pathogenic mechanisms underlying a variety of human diseases, ranging from viral infections to various forms of cancer. Improved comprehension of the pathophysiology of disease entities has, in turn, fostered the development of stratagems designed to specifically interrupt or correct disease processes at the molecular level. Thus, gene transfer or gene therapeutic approaches are being used to study the biologic behavior of specific genetically marked cell populations (8), to augment the functions of defective genes (e.g., genes causing adenosine deaminase deficiency or hemoglobinopathies) (3, 6, 7, 52), and to interrupt the viral life cycle (10, 48, 51, 54, 55) or oncogenesis (22, 46) (for reviews see references 1, 33, 35, 37, and 53 and references therein). Various methods have recently been developed for in vitro gene transfer; however, few of these possess the high efficiency necessary for in vivo therapeutic use. One elegant strategy for gene delivery exploits the natural ability of viruses to stably transfer their genetic material into cells. Indeed, vectors derived from retroviruses have been used clinically, to insert the wild-type adenosine deaminase gene into T lymphocytes of individuals with severe combined immunodeficiency disease (6) and to deliver marker genes to study the behavior of specific cellular populations in vivo (8). However, retroviruses and retrovirus-based vectors require actively proliferating target cells for successful infection and proviral integration (16, 19, 34, 47). This requirement for actively proliferating target cells presents a major impediment for gene transfer into certain attractive targets of gene therapy, such as T lymphocytes and hematopoietic stem cells. Human T lymphocytes have been successfully used as targets for retrovirus-mediated gene modification in clinical trials. Normally, this population of cells is quiescent (14) and retroviral transduction requires T-cell activation (47). However, for purposes of gene therapy for human immunodeficiency type 1 (HIV-1) infection, stimulation to induce proliferation, such as cytokine treatment, may result in activation of latent virus and may render uninfected cells susceptible to productive infection with HIV, thus accelerating the course of the disease. Similarly, since totipotent hematopoietic stem cells are postulated to be quiescent (40, 50), the inability to transfer genes into nondividing cells has been a major impediment to stem cell transduction. For example, in mice, only the quiescent population of marrow-derived cells contain sufficient stem cells to transfer long-term hematopoietic reconstitution to lethally irradiated secondary recipients (50). Although cytokine activation of hematopoietic stem cells renders them accessible to retroviral transduction, such treatment also commits cells to certain differentiation pathways, thus altering the self-renewal capacity and the ultimate fate of transduced cells (40). Thus, the inability to transfer genes stably and efficiently into nondividing cells represents a significant hindrance to the field of gene therapy.

We have recently reported the use of viral vectors based on the nonpathogenic parvovirus adeno-associated virus (AAV) to efficiently confer resistance to HIV-1 and herpes simplex virus type 1 (HSV-1) (10, 54). AAV is a single-stranded, replication-defective DNA virus with a 4.7-kb genome with palindromic inverted terminal repeats (4). Coinfection with a helper virus, such as adenovirus or HSV, is required for productive infection (9, 25). Molecular clones containing the entire AAV-2 genome are infectious following transfection into helper virus-infected cells, facilitating genetic manipulation. In the absence of helper virus coinfection, AAV integrates in a stable fashion via the inverted terminal repeats into cellular DNA (5). In addition, wild-type AAV has recently

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been shown to integrate in a site-specific manner into the q arm of human chromosome 19 (26, 27, 44). The possibility of directing similar site-specific integration of AAV-based vectors is attractive for gene therapeutic use, as it would minimize the risk of insertional mutagenesis and the variability of transgene expression. Genes inserted into AAV vectors may be precisely designed to direct synthesis of short, defined transcripts (11). AAV vectors have high transduction frequencies (21, 29, 32, 36, 49) in cells of diverse lineages, including hematopoietic cells, which are attractive targets for ex vivo human gene therapy (12, 28, 57). Additionally, AAV vectors often integrate in tandem in multiple copies, thereby enhancing transgene expression. Finally, latent wild-type AAV infections have been stably maintained in tissue culture for over 100 serial passages in the absence of selective pressure, attesting to the stability of AAV genomic integration (5). Thus, AAV vectors are well suited for the stable introduction of transgenes into human cells.

In this study, we analyzed the feasibility and efficacy of utilizing AAV vectors to transfer genes into nondividing cells. An AAV vector encoding the bacterial β-galactosidase gene under control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (vCWR:βGal) was constructed and encapsidated. vCWR:βGal was used to compare AAV-mediated gene transfer in vitro into actively replicating cells, cells arrested in mitosis by treatment with the DNA synthesis inhibitors fluorodeoxyuridine (FUdR) (20) and aphidicolin (45), and human diploid fibroblasts induced into quiescence by confluence and propagation in low-serum medium. The stability of vector integration following AAV transduction of noncycling cell populations was determined by hybridization analysis of vector-specific sequences within high-molecular-weight genomic DNA isolated from cells passaged in culture following a 100,000-fold expansion after transduction. Our results suggest that AAV-based vectors are capable of efficient transduction of nondividing cells and that vector sequences introduced into nondividing cells can ultimately integrate in the absence of selective pressure.

## MATERIALS AND METHODS

**Cells and viruses.** 293-31 cells (17), a human embryonic kidney line transformed with adenovirus type 5 DNA, were passaged in calcium- and magnesium-free Eagle's minimal essential medium with twice the normal concentrations of vitamins and amino acids (Quality Biologicals, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO, Grand Island, N.Y.) and 2 mM glutamine as previously described (10, 11). Diploid human foreskin fibroblasts (HFF) (56) and the human diploid fibroblast cell line MRC-5 (24) (American Type Culture Collection) were passaged in Dulbecco modified Eagle medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% FCS. Both cell lines represent normal untransformed human cells of finite life span and were used at low passage number. HSV-1 MP17 was propagated and titered by plaque assays on Vero cells as previously described (9, 54). All cells were tested and proven to be free of mycoplasma contamination. Cells were routinely cultured at 37°C in humidified 5% $CO_2$.

**vCWR:βGAL construction and encapsidation.** vCWR:βGal was constructed by using standard cloning methods (43). Briefly, the 484-bp *Sal*I-*Sna*BI fragment was removed from pCWRSV, an AAV-2-based vector containing the endogenous AAV polyadenylation signal cloned within a high-copy-number pUC-based plasmid (11), and a synthetic 44-bp oligonucleotide polylinker was inserted to yield pCWR-PA. The β-galactosidase gene with a simian virus 40 (SV40) polyadenylation signal was isolated as a 3,749-bp *Hin*dIII-*Sal*I fragment from pSVβGal and inserted 3′ to the RSV promoter into pCWR-PA to yield pCWR:βGal (Fig. 1). The integrity of AAV inverted terminal repeats, critical for AAV origin-dependent replication, vector encapsidation, and chromosomal integration, was confirmed by *Msc*I and *Sma*I digestions followed by agarose gel analysis. Recombinant plasmids were purified by cesium chloride gradient centrifugation as previously described (43).

Recombinant pCWR:βGal was encapsidated by previously described methods (10, 11). Briefly, semiconfluent 293 cells were grown in 100-mm-diameter dishes and infected with HSV-1 MP17 (multiplicity of infection [MOI] of 0.1). Cells were transfected 1 h postinfection with 15 μg of pCWR:βGal and 3 μg of pTAAV, a plasmid providing AAV-encoded DNA replication and encapsidation gene functions in *trans* (10, 11). Transfections were performed by calcium phosphate coprecipitation (CellPhect; Pharmacia Biotech, Uppsala, Sweden). Cells were harvested 48 h posttransfection and lysed, and the residual HSV-1 was heat inactivated. Recombinant AAV-vector stocks were heat inactivated until HSV-1-mediated cytopathic effects were not detectable, at which time aliquots were plated onto susceptible 293 cells, generally about 6 to 9 h at 56°C.

The titer of the encapsidated vector (vCWR:βGal) was determined by serial dilution onto 293 cells and enumeration of β-galactosidase-expressing cells, determined by staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Bluogal; Gibco-BRL, Gaithersburg, Md.) as described below. vCWR:βGal titers routinely ranged between $10^3$ and $10^5$ transducing units/ml, slightly lower than for other vectors encapsidated similarly. Wild-type AAV was not detected in the encapsidated vector stocks, as determined by hybridization to wild-type AAV-specific probes.

**Mitotic arrest and quiescence.** Cells were mitotically arrested by (i) treatment with the DNA synthesis inhibitor FUdR or the DNA polymerase alpha inhibitor aphidicolin and (ii) contact inhibition with propagation in low-serum medium. The optimal concentrations of FUdR and aphidicolin necessary to efficiently inhibit cell proliferation were initially determined. 293 cells were seeded at $5 \times 10^3$ cells per well in triplicate in 96-well plates and allowed to adhere overnight. The following day, the medium was replaced with fresh medium containing FUdR (Quad Pharmaceuticals, Indianapolis, Ind.) at 1, 5, 10, 20, 50, and 100 nM or aphidicolin (Sigma, St. Louis, Mo.) at 1, 5, 10, and 20 μg/ml. Cells were treated for 24 h, extensively washed, and analyzed for viability by trypan blue dye exclusion and for DNA synthesis by tritiated deoxycytidine ([$^3$H]dCTP) incorporation as follows (Fig. 2). One microcurie of [$^3$H]dCTP (18 Ci/mmol; ICN Biomedicals, Costa Mesa, Calif.) was added to each well of triplicate cultures and incubated in medium containing 10% dialyzed FCS for 4 h. Cells were harvested with a PHD Harvester (Cambridge Technologies, Cambridge, Mass.) and washed eight times, and $^3$H incorporation was measured by scintillation counting. [$^3$H]dCTP was used to measure DNA synthesis because, in contrast to thymidine, its metabolism is less likely to be influenced by the action of FUdR (20). FUdR at 20 nM and aphidicolin at 5 μg/ml were found to optimally inhibit 293 cell proliferation, and these concentrations were used in subsequent studies to evaluate AAV-mediated gene transfer.

Mitotic arrest of drug-treated cells was confirmed by quantitation of cell division rate, flow cytometric analysis, and determination of [$^3$H]dCTP incorporation at the time of vector transductions (Fig. 2). The division rate of 293 cells was

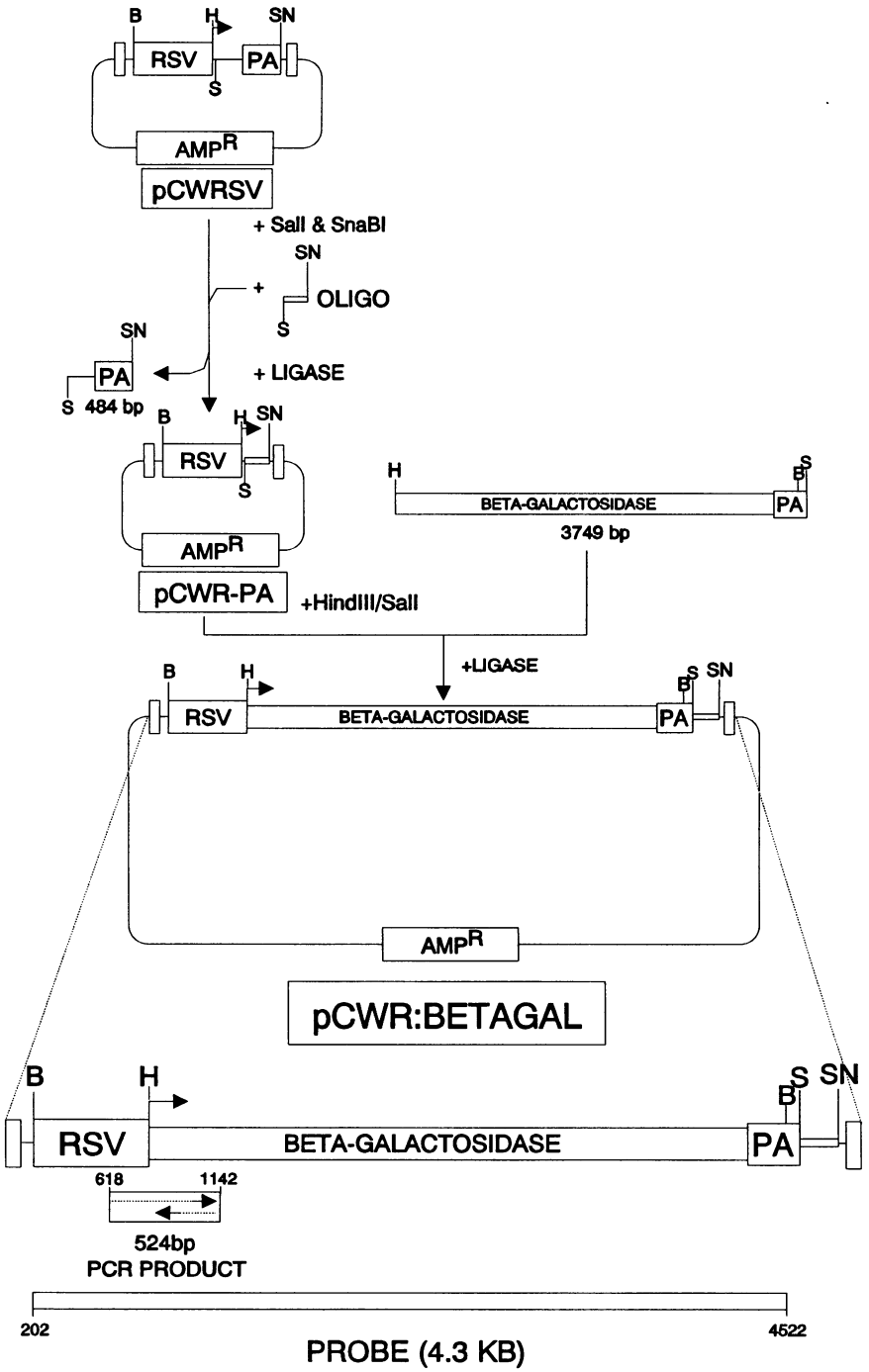


FIG. 1. CWR:βGal vector construction, PCR primers, and probe sequences. The β-galactosidase gene with an SV40 polyadenylation signal (PA) was isolated as a 3,749-bp *Hin*dIII-*Sal*I fragment and inserted under RSV promoter control into pCWR-PA to yield pCWR:βGal. The regions of CWR:βGal corresponding to PCR primers and the $^{32}$P-labeled probe are depicted. B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; SN, *Sna*BI; OLIGO, oligonucleotide.

determined by seeding triplicate 96-well plates at approximately $5 \times 10^3$ cells per well and treating them with FUdR or aphidicolin for 24 h (day 1) as described above. On day 2, drug-treated cells and untreated controls were washed twice with phosphate-buffered saline (PBS; Irvine Scientific), overlaid with complete medium, and incubated at 37°C in 5% $CO_2$. Cell counts and viability were determined on days 1, 2, and 4. [$^3$H]dCTP incorporation was assayed as described above. Flow cytometric studies using propidium iodide (18) were done on day 2 after removal of FUdR to determine the DNA content and, thus, the cell cycle status of the population. Briefly, cells were treated with DNase-free RNase (150 μg/$10^6$ cells) at 37°C

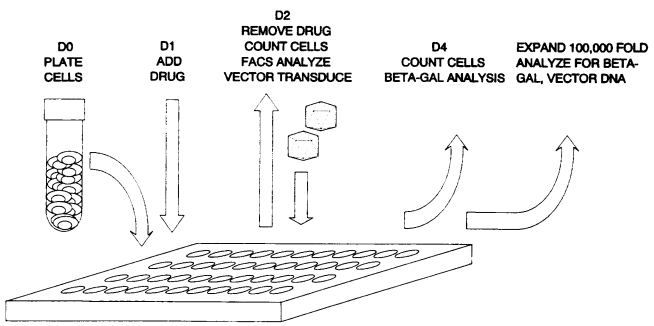


FIG. 2. Experimental scheme for growth arrest and transduction of 293 cells. Cells were treated with either 20 nM FUdR or 5 μg of aphidicolin per ml for 24 h (day 1 (D1), which was then washed out. On day 2, cells were analyzed for $^3$H-NTP incorporation and for flow cytometric profiles of DNA content and were transduced with vCWR: βgal. Cell counts and viability were determined on days 1, 2, and 4. β-Galactosidase (BETA-GAL) expression was assayed on day 4. In addition, approximately 200 untreated or drug-treated cells were transduced and expanded for approximately 2 months prior to analysis for the presence of vector-specific DNA by PCR and Southern blots. FACS, fluorescence-activated cell sorting.

for 30 min, washed once with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2)–0.1% bovine serum albumin (BSA), stained in the dark with 100 μg of propidium iodide (Calbiochem, La Jolla, Calif.) per ml in 10 mM HEPES–0.1% BSA for 16 h at room temperature, and analyzed by flow cytometry (Becton Dickinson FACS IV).

Diploid HFF and MRC-5 cells were induced into quiescence by growth to 100% confluence in Dulbecco modified Eagle medium containing 10% FCS and then maintained in medium containing 5% FCS. The medium was replaced every 3 days with low-serum media for 14 days prior to analysis. Previous reports have shown that these conditions effectively produce quiescence in these cells (34). Actively proliferating controls were harvested and replated in medium containing 10% FCS at 60% confluency 1 day prior to analysis ($2 \times 10^4$ to $3.5 \times 10^4$ cells per well). Cell counts and viability were determined as described above. In these experiments, [$^3$H]TTP (65 Ci/mmol; ICN Biomedicals) was used to measure DNA synthesis in a 7-h uptake.

**vCWR:βGAL transduction of actively dividing and growth-arrested cells.** 293 cells, in triplicate, were treated with either FUdR or aphidicolin for 24 h. Untreated, subconfluent cells served as actively proliferating controls. Monolayers were washed twice with PBS at the completion of drug treatment, and 100 μl of fresh medium without drug was added to each well. vCWR:βGal was added at an MOI of approximately 1 and adsorbed for 1 h at 37°C, and then residual free vector was removed by washing with PBS. Fresh medium was then added to the transduced monolayers, and the cultures were incubated as described above (Fig. 2). Transduced cells were assayed for β-galactosidase expression at 48 h posttransduction as described above. Briefly, cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde–1 mM $MgSO_4$–100 mM sodium phosphate (pH 7.2), and washed three times in PBS. Fixed cells were then incubated in X-Gal (432 μg of X-Gal per ml in 3.1 mM potassium ferricyanide–3.1 mM potassium ferrocyanide–10 mM sodium phosphate buffer [pH 7.2]–0.15 M NaCl–1.0 mM $MgCl_2$) at 200 μl per well for 16 h, and β-galactosidase-positive cells were enumerated by microscopic analysis.

HFF and MRC-5 human diploid fibroblast cells were propagated in low-serum medium for 14 days after reaching 100% confluence. Corresponding cells in logarithmic growth were plated at 60% confluency 1 day prior to transduction and served as actively proliferating controls. Quiescent and proliferating HFF and MRC-5 cells were transduced with vCWR: βGal at an MOI of 1, and β-galactosidase expression was assayed as described above. Quiescent cells were propagated in low serum throughout the period following transduction to perpetuate the proliferative block.

**Integration analysis of vCWR:βGAL into mitotically arrested and dividing 293 cells.** Integration of vector sequences was analyzed in untreated or FUdR- or aphidicolin-treated 293 cells. Two hundred cells from each treatment group were transduced with vCWR:βGal at an MOI of 1 in replicate cultures as described above. Transduced cells were either assayed for β-galactosidase expression or serially expanded into progressively larger tissue culture vessels over a period of approximately 2 months. The total number of expanded cells was determined after each passage. Cells were harvested for DNA analysis when they had expanded to a total of $2 \times 10^7$ cells, representing a 100,000-fold increase. Cells were harvested, washed, lysed, and treated sequentially with DNase-free RNase A at 300 μg/ml and proteinase K at 1 mg/ml. High-molecular-weight genomic DNA was extracted by using anion-exchange chromatography columns (A.S.A.P.; Boehringer Mannheim, Indianapolis, Ind.) as instructed by the manufacturer, and DNA concentrations and purity were determined spectrophotometrically. The presence of integrated vector sequences was determined by PCR amplification utilizing vector-specific primers in a Perkin-Elmer model 9600 Thermal Cycler. One microgram of genomic DNA template was subjected to 30 cycles of amplification (denaturing at 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 1 min, with a final extension for 5 min at 72°C). Oligonucleotides were synthesized on an Applied Biosystems model 391 synthesizer (Applied Biosystems, Foster City, Calif.). Vector-specific primers (sense, 5'-GGTGGAAGTAAGGTG GTACG-3'; antisense, 5'-GCTGCAAGGCGATTAAGTTG -3') were used to amplify a 524-bp product spanning a region from the RSV LTR to the β-galactosidase gene (nucleotides 618 to 1142) (Fig. 1). An internal control for template integrity was provided by amplification of a 268-bp fragment from the ubiquitous human β-globin gene (sense primer, 5'-CAACT TCATCCACGTTCACC; antisense primer, 5'-GAAGAGC CAAGGACAGGTAC). Amplified products were resolved by electrophoresis in a 1.4% agarose gel in Tris acetate-EDTA buffer (TAE) (43), transferred to supported nitrocellulose (Schleicher & Schuell, Keene, N.H.), and hybridized to a 4.3-kb *Bam*HI $^{32}$P-random-primer-labeled, high-specific-activity probe (Decaprime II, Ambion, Tex.). The probe corresponded to RSV LTR and β-galactosidase coding sequences (nucleotides 202 to 4522; Fig. 1). Hybridization intensity was quantitated by gas scanning (Ambis, San Diego, Calif.).

For Southern hybridization analysis, 20 μg of high-molecular-weight genomic DNA extracted from cells transduced while in logarithmic growth, after aphidicolin or FUdR treatment, and from untransduced cells was digested with *Sca*I or *Sac*I, restriction enzymes which either do not cleave or cleave once within the vector sequence, respectively. Restriction digest fragments were resolved by electrophoresis in a 0.8% agarose gel in TAE, transferred to supported nitrocellulose, and hybridized to the 4.3-kb *Bam*HI $^{32}$P-labeled vector-specific probe described above (Fig. 1).

## RESULTS

**Profound but reversible growth arrest of 293 cells with FUdR.** The general schema of mitotic arrest experiments is depicted in Fig. 2. To induce mitotic arrest in 293 cells, we treated cells with FUdR, a potent inhibitor of DNA synthesis that has previously been used to study the cell cycle (20). Initial titration experiments revealed a concentration of 20 nM to be optimal for the induction of cell cycle block without significant cytotoxicity. To determine the extent of mitotic arrest induced by FUdR, we compared the cell division rate of FUdR-treated cells with that of logarithmically growing controls. As shown in Fig. 3A, the number of untreated, actively replicating 293 cells increased 100% by day 2. In contrast, the number of FUdR-treated cells showed no increase during the same period. However, by day 4 (2 days after removal of FUdR from the culture), the number of FUdR-treated cells had more than doubled over day 1 values, indicating that removal of the drug allowed the cells to recover from growth arrest and resume active proliferation. Thus, treatment of 293 cells with 20 nM FUdR for 24 h resulted in a profound but reversible inhibition of cell proliferation in comparison with untreated, logarithmically growing controls.

Flow cytometric studies were performed on cells fixed and stained with propidium iodide on day 2 to determine the DNA content and proliferating status of the FUdR-treated and control cell populations. Figure 3B and C show 50% of cells in $G_0/G_1$, 16% in $G_2/M$, and 34% in S phase in the untreated controls (Fig. 3B), compared with 19% in $G_0/G_1$, 12% in $G_2/M$, and 69% in S phase in the FUdR-treated cells (Fig. 3C). The increase in the relative proportion of cells in S phase in the FUdR-treated population confirmed an S-phase block. Untreated cells displayed the expected distribution of cells in the different mitotic phases.

Lastly, [$^3$H]dCTP incorporation into cellular DNA was measured to determine the rate of DNA synthesis in FUdR-treated cells compared with controls (Table 1). An 88% reduction of [$^3$H]dCTP incorporation was observed in FUdR-treated cells relative to actively proliferating controls on day 2. These results suggested that FUdR treatment of 293 cells for 24 h resulted in a significant S-phase arrest.

**vCWR:βGal transduction of actively dividing and FUdR-treated, growth-arrested 293 cells.** FUdR-treated cells confirmed to be in mitotic arrest and logarithmically growing controls were transduced with vCWR:βGal at an MOI of 1. Transductions were performed on day 2 (Fig. 2) in cultures parallel to those described above for analysis of cell proliferation, flow cytometry, and [$^3$H]dCTP uptake. Thus, the proliferative status of cells at the time of transduction was identical to that of cells confirmed to be mitotically arrested as described above. Transduction efficiency was measured by quantitating the fraction of cells expressing the β-galactosidase gene 48 h posttransduction. The background level of β-galactosidase expression in untransduced cells was always zero. Results from two independent experiments performed in triplicate at an MOI of 1 are shown in Fig. 3D. In both experiments, the transduction efficiency of growth-arrested cells was at least equivalent to that of actively proliferating controls. Similar results were obtained when cells were transduced and maintained in the presence of FUdR for the entire 48-h assay period. Interestingly, these and other experiments noted below revealed vCWR:βGal transduction to be marginally greater in the nonproliferating than the logarithmically proliferating cell populations. Any potential biological significance of this observation is currently unclear.

**Profound and sustained growth arrest of 293 cells with aphidicolin.** To confirm the universality of the foregoing findings of gene transfer into nondividing cells, we tested AAV-mediated gene transfer into 293 cells treated with aphidicolin, a DNA polymerase alpha inhibitor. Unlike FUdR treatment, a 24-h treatment with aphidicolin at 5 μg/ml induced a sustained mitotic arrest in 293 cells, as determined by measurement of cellular proliferation after removal of the drug (Fig. 4A). Aphidicolin-treated cells showed no increase in cell numbers compared with control proliferating cells for at least 5 days after removal of the drug from culture (data not shown).

The DNA synthetic activity of aphidicolin-treated cells was measured by analyzing [$^3$H]dCTP incorporation on day 2 (Table 1). [$^3$H]dCTP incorporation was reduced by 97% in aphidicolin-treated cells compared with exponentially replicating controls (Table 1). These results confirmed that aphidicolin-treated cells were truly arrested in division.

**vCWR:βGal transduction of dividing and aphidicolin-treated, growth-arrested 293 cells.** We next determined the efficacy of AAV-mediated gene transfer into actively proliferating and aphidicolin-treated, mitotically arrested cells. Cells were treated with aphidicolin for 24 h and transduced with vCWR:βGal at an MOI of 1 immediately after removal of the drug (Fig. 2). Thus, vector-transduced control and aphidicolin-treated cells were identical to those analyzed above for cell proliferation and DNA synthesis. Results of two independent experiments, each performed in triplicate, are shown in Fig. 4B. As above, transduction efficiency was assayed by β-galactosidase expression 48 h after transduction. Once again, the number of β-galactosidase-expressing cells was not only equivalent but slightly higher in mitotically arrested cells compared with actively proliferating controls.

**Growth arrest of human diploid fibroblast cell lines by contact inhibition and maintenance in low-serum medium.** To test AAV-mediated gene transfer into nondividing diploid cells in the absence of chemical inhibitors of DNA synthesis, we used two primary, diploid fibroblast cell lines, HFF and MRC-5, which exhibit contact inhibition and therefore may be induced into a nonreplicative phase without the use of mitotic inhibitors. We reasoned that this strategy may more closely simulate in vivo conditions for gene transfer. Both HFF and MRC-5 cells were grown to 100% confluency in low serum concentrations for 14 days prior to analysis. Cells remained viable but quiescent throughout this period. Subconfluent cells, plated 24 h prior to analysis, served as proliferating controls. Measurement of [$^3$H]TTP incorporation confirmed the nonreplicative status of contact-inhibited cells; [$^3$H]TTP uptake was reduced by 95 and 92% in comparison with actively replicating controls of HFF and MRC-5 cells, respectively (Table 1).

**vCWR:βGal transduction of growth-arrested human fibroblasts.** Contact-inhibited and actively replicating HFF and MRC-5 cells (as determined by [$^3$H]TTP incorporation; see above) were transduced with vCWR:βGal at an MOI of 1, and β-galactosidase expression was determined as previously described. Detection of β-galactosidase activity following transduction of these two primary diploid fibroblast lines was substantially lower than that observed in other cell lines. The reason for this low-level expression in these cell lines is unclear. Nevertheless, specific β-galactosidase staining was detected, while untransduced controls showed no background staining for endogenous β-galactosidase. Figure 5 shows β-galactosidase expression in vector-transduced, nonproliferating cells was again not only equivalent to but greater than that in actively proliferating HFF and MRC-5 controls.

**Stable integration of vCWR:βGal sequences in growth-**

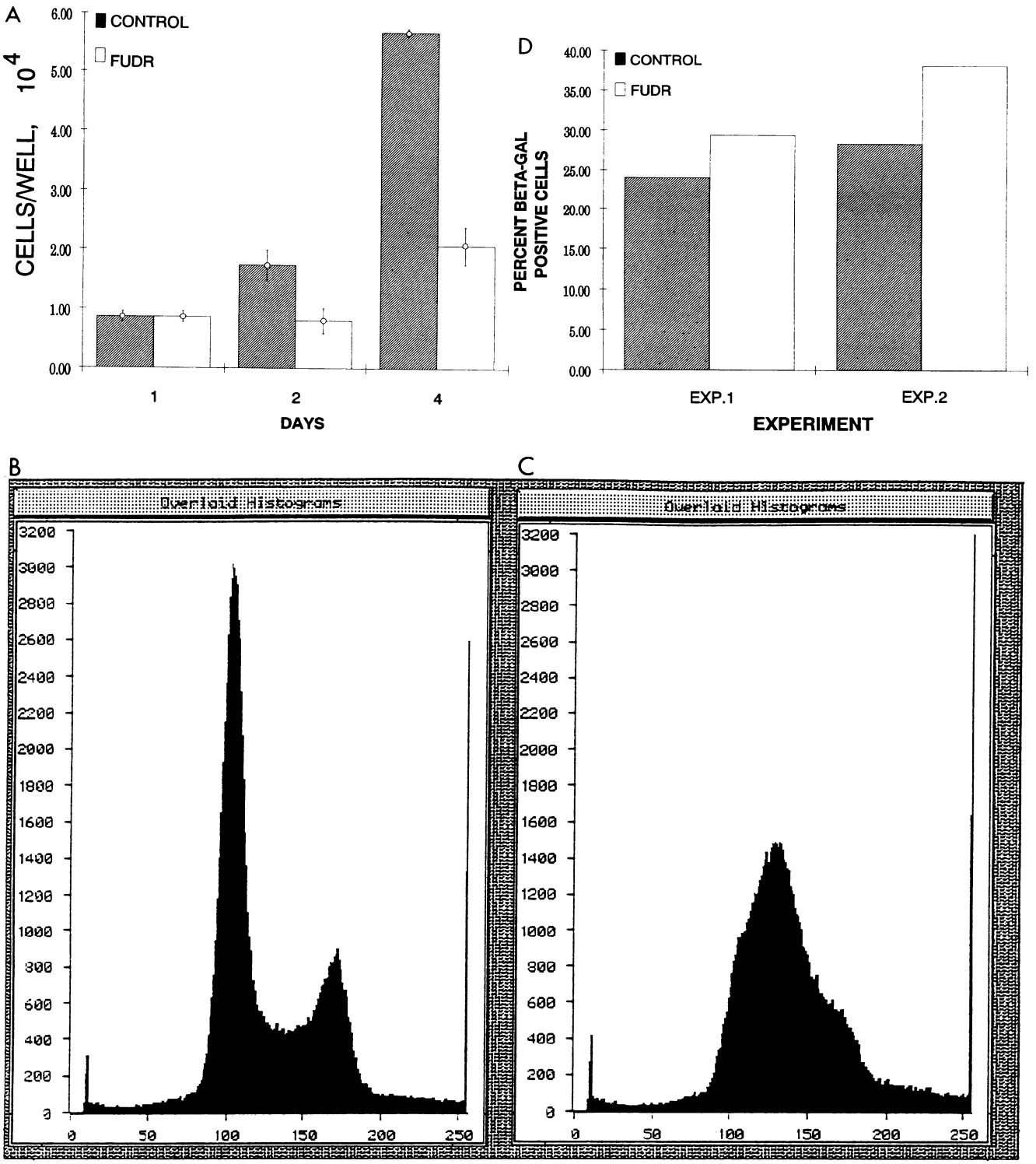


FIG. 3. vCWR:βGal transduction of cells arrested in mitosis with FUdR. (A) 293 cells were treated with 20 nM FUdR for 24 h prior to extensive washes and replacement of the medium. Results shown are the means and standard deviations of transductions performed in triplicate on days 1, 2, and 4 after FUdR treatment. (B and C) Flow cytometric analysis of cellular DNA content of 293 cells in logarithmic growth (B) or FUdR-treated 293 cells (C). The *x* axis shows the relative DNA content per cell; the *y* axis shows the cell number per channel. Channels 94 to 113, cells in $G_0/G_1$; channels 113 to 164, cells in S phase; channels 164 to 183, cells in $G_2/M$. (D) β-Galactosidase (BETA-GAL) expression in FUdR-treated and logarithmically growing 293 cells transduced with vCWR:βGal at an MOI of 1.

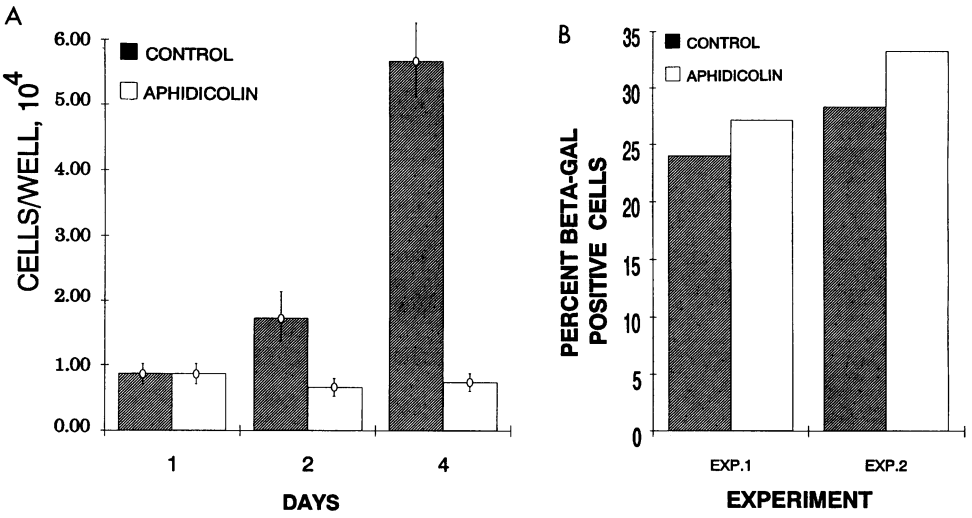


FIG. 4. vCWR:βGal transduction of cells arrested in DNA synthesis by aphidicolin. (A) 293 cells were treated with 5 μg of aphidicolin per ml for 24 h prior to extensive washes and replacement of the medium. Cell counts were performed in triplicate on days 1, 2, and 4. Shown are means and standard deviations of triplicate samples. (B) β-Galactosidase (BETA-GAL) expression in actively replicating and aphidicolin-treated 293 cells transduced with vCWR:βGal at an MOI of 1.

**arrested and dividing 293 cells in the absence of selective pressure.** We next tested whether AAV-mediated gene transfer into nonproliferating cells actually resulted in the ultimate stable integration of vector sequences into cellular chromosomal DNA. Actively proliferating, FUdR-treated and aphidicolin-treated 293 cells were transduced with vCWR:βGal (MOI of 1). Transduction of FUdR- or aphidicolin-treated cells was performed during mitotic arrest as described above. Following transduction, the mitotic block was removed and cells were serially passaged in the absence of selective pressure, with cell counts being performed at the time of transduction and during each subsequent passage. Chromosomal DNA from these cells was extracted after a 100,000-fold expansion in cell numbers posttransduction, approximately representing an 8-week period. β-Galactosidase expression was detected in transduced cells over a period of 9 months of cellular expansion. High-molecular-weight genomic DNA was isolated and analyzed for the presence of vector DNA both by PCR amplification using vector-specific primers (see Fig. 1 for locations of primers and probes) and by genomic Southern hybridizations.

Amplification of vector-specific sequences followed by hybridization of the amplified products with a vector-specific probe revealed the presence of the expected 524-bp product in both transduced FUdR- or aphidicolin-treated and replicating cell DNA but not in untransduced controls (Fig. 6A). Analysis of the intensity of hybridization of the $^{32}$P-labeled, vector-specific probe revealed the presence of similar amounts of vector-specific product in transduced growth-arrested cells compared with actively replicating controls. The amplification of an appropriately sized 268-bp β-globin fragment from all four DNA templates with β-globin-specific primers controlled for DNA template integrity (Fig. 6B).

To confirm that the vector sequences detected in high-molecular-weight cellular DNA represented integrated forms of the vector, DNA was extracted from cells transduced while in logarithmic growth or mitotic arrest and from untransduced cells. Digestion of genomic DNA containing integrated vector sequences with a noncutting restriction enzyme would be expected to produce fragments larger than the vector itself (4.8 kb). Southern blot analysis of vector-transduced genomic DNA following digestion with *Sca*I, a restriction enzyme that does not cleave within vector sequences, is depicted in Fig. 7. A

TABLE 1. Reduction of $^3$H-dNTP uptake in growth-arrested cells

| Cell type | Treatment | % Reduction of $^3$H incorporation[a] |
|---|---|---|
| 293 | FUdR | 88 |
| 293 | Aphidicolin | 97 |
| HFF | Confluence, low serum | 95 |
| MRC-5 | Confluence, low serum | 92 |

[a] Normalized to equivalent numbers of actively replicating control cells. Percentages represent the averages of triplicate values.

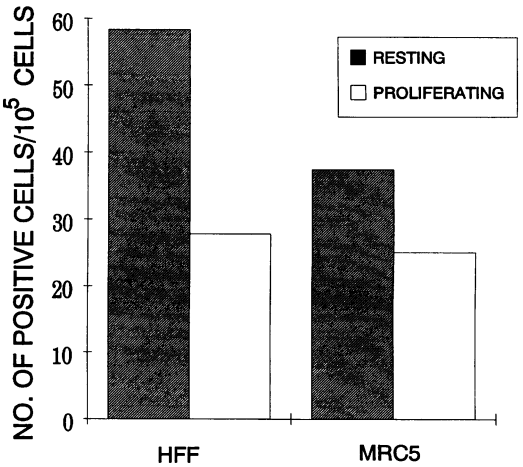


FIG. 5. vCWR:βGal expression in growth-arrested diploid human fibroblast lines HFF and MRC-5. HFF and MRC-5 cells were induced into quiescence by contact inhibition and maintained in low-serum medium for 2 weeks prior to transduction with vCWR:βGal.

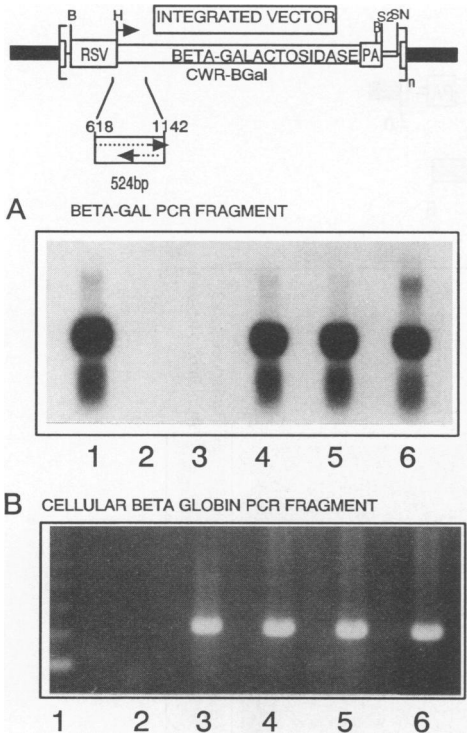


FIG. 6. Integration analysis of vCWR:βGal genomes into replicating and growth-arrested 293 cells. Genomic DNA was extracted from vCWR:βGal-transduced actively replicating and growth-arrested cells following a 100,000-fold expansion. (A) A 524-bp fragment specific for vCWR:βGal was amplified from transduced dividing and mitotically arrested cells but not from untransduced controls, using vector-specific primers (the locations of primers are depicted [PA, SV40 polyadenylation signal; B, *Bam*HI; S2, *Sal*I site within vector; n, potential tandem repeats of integrated vector sequences]). Amplified products were resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a $^{32}$P-labeled vector-specific probe. Lanes: 1, positive plasmid control; 2, no-template DNA control; 3, untransduced 293 cells; 4, transduced actively replicating 293 cells; 5, FUdR-treated vCWR:βGal-transduced 293 cells; 6, aphidicolin-treated vCWR:βGal-transduced 293 cells. BETA-GAL, β-galactosidase. (B) Amplification of a 268-bp β-globin gene fragment from the DNA samples used in panel A, demonstrating the integrity of the DNA templates. Lanes: 1, molecular weight marker; 2, no-template control; 3, untransduced 293 cells; 4, transduced actively replicating 293 cells; 5, FUdR-treated vCWR:βGal-transduced 293 cells; 6, aphidicolin-treated vCWR:βGal-transduced 293 cells.

predominant vector-specific band of approximately 9 kb is demonstrable in *Sca*I-digested DNA from both aphidicolin-treated and logarithmically growing 293 cells. To differentiate between high-molecular-weight unintegrated concatemeric vector sequences and true vector integration into cellular DNA, an aliquot of genomic DNA was digested with *Sac*I, which cleaves once within the vector at base 3011. *Sac*I cleavage should resolve unintegrated unit-length vector into fragments of 3 and 1.8 kb, unintegrated concatemers into unit-length vector (4.8 kb) and fragments of 3 and 1.8 kb, and integrated concatemers into unit-length vector and junction fragments consisting of vector and cellular DNA, while single-copy integrants should be cleaved into two fragments, the sizes of which would depend upon *Sac*I cleavage sites in the flanking chromosomal sequences. Figure 7B depicts the Southern blot analysis of such an experiment. Vector-specific fragments of approximately 8 and 2.5 kb are demonstrated, strongly supporting vector integration. The presence of similarly sized bands in actively dividing 293 cells and in 293 cells transduced while in FUdR- or aphidicolin-induced DNA synthesis arrest suggests that regardless of the cell division status at the time of transduction, the ultimate intracellular fate of the vector sequences is the same under each condition tested. Furthermore Southern hybridization of undigested DNA harvested from aphidicolin-treated cells 96 h posttransduction did not result in the appearance of vector-specific bands (data not shown), again indicating the absence of free vector sequences.

## DISCUSSION

In this study, we demonstrated the ability of an AAV-based vector (vCWR:βGal) to mediate stable transduction of cells arrested in mitosis. Equivalent levels of transduction by vCWR:βGal into 293 cells blocked in mitosis by two different inhibitors of DNA synthesis, FUdR and aphidicolin, compared with actively dividing cells indicated that AAV-mediated gene transfer into nondividing cells was not drug specific. Similarly, transduction of two untransformed diploid human fibroblast cell lines induced into quiescence by confluence and maintenance in low serum was no less than that of cells in logarithmic growth. In each instance, the nondividing status of cells was verified by at least two independent methods, cell proliferation and $^{3}$H-labeled deoxynucleoside triphosphate (dNTP) uptake, and in some instances, S-phase arrest was also confirmed by flow cytometric analysis. In each case, the number of transduced cells in the mitotically arrested population was observed to be equivalent to and frequently greater than that of actively dividing cells. Whether this was due to a greater accumulation of the β-galactosidase enzyme in nondividing cells or to other mechanisms affecting the efficiency of vector transduction is unclear.

Although FUdR treatment of 293 cells for 24 h resulted in a profound inhibition of cell proliferation and DNA synthesis by all parameters tested, the mitotic block was reversible, and cells recovered over a period of 24 to 72 h after removal of the drug. Thus, vCWR:βGal transduction of a small fraction of recovering, proliferating 293 cells could not be excluded. Therefore, we tested AAV-mediated gene transfer following a more complete and profound mitotic arrest induced by aphidicolin. In contrast to FUdR treatment, the mitotic block induced by aphidicolin lasted at least 5 days following removal of the drug, with insignificant $^{3}$H-dNTP incorporation and no detectable cellular proliferation at the time of transduction. However, once again, vCWR:βGal transduction of aphidicolin-treated cells was equivalent to that in the actively replicating controls. Finally, vCWR:βGal transduction of quiescent diploid fibroblasts confirmed the ability of AAV-based vectors to transduce cells which may be more representative of physiologically quiescent ($G_0$) cells in vivo. Contrary to FUdR-induced growth arrest, the proliferative block in the primary diploid cells, mediated by contact inhibition, was present 14 days prior to vector transduction and persisted until cells were tested for β-galactosidase expression 48 h later. Therefore, we conclude that AAV-mediated transduction could occur in the milieu of a prolonged absence of DNA synthesis.

Hybridization analysis of high-molecular-weight genomic DNA isolated after a 100,000-fold expansion posttransduction indicated that vector integration into cellular chromosomal DNA was stable and could be maintained in the absence of selective pressure. Indeed, wild-type AAV sequences have been reported to exist in a stably integrated form for over 100 passages in the absence of selection (5). Our result, however,

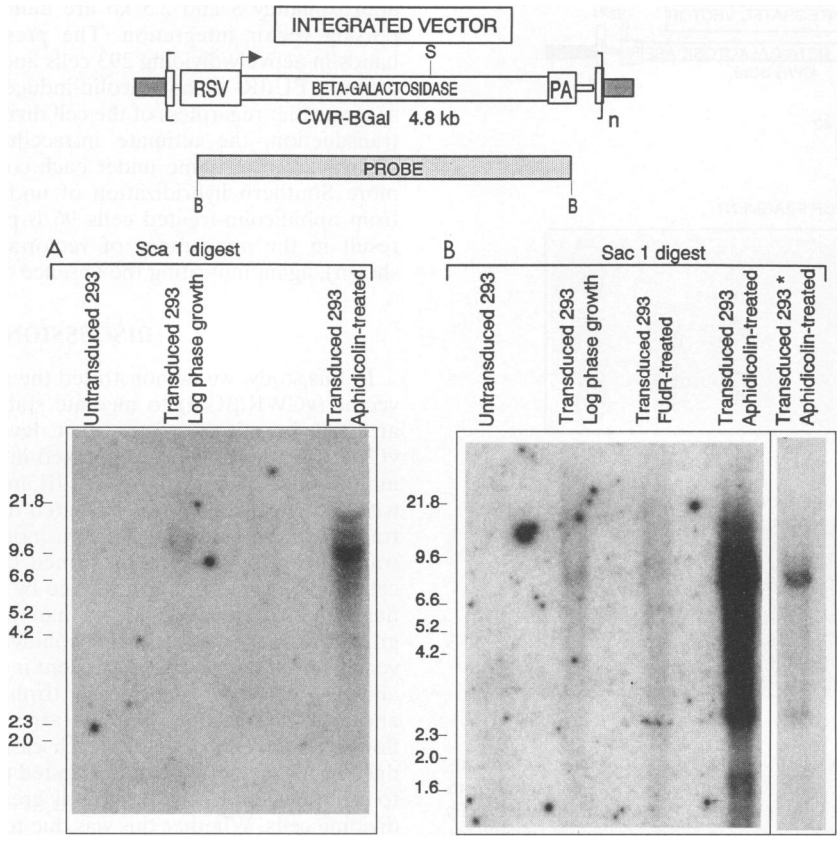


FIG. 7. Southern hybridization analysis of genomic DNA from replicating and growth-arrested 293 cells digested with *Sca*I, which does not cleave within vector sequences, and *Sac*I (S), which cleaves once within the vector. Twenty micrograms of digested genomic DNA was electrophoresed per lane in 0.8% agarose in TAE buffer, transferred to nitrocellulose, and hybridized with a vector-specific probe (a *Bam*HI fragment depicted at the top; for designations, see the legend to Fig. 6). Shown are hybridization analyses of restriction digests with *Sca*I (A) and *Sac*I (B) of DNA from untransduced 293 cells, vCWR:βGal-transduced, actively replicating 293 cells, vCWR:βGal-transduced, FUdR-treated 293 cells (*Sac*I only), and vCWR:βGal-transduced, aphidicolin-treated 293 cells. *, shorter exposure of aphidicolin-treated 293 cell DNA. Sizes are indicated in kilobases.

does not distinguish between the possibility of vector integration into chromosomal DNA of nondividing cells versus the persistence of the vector in an episomal form which integrates after release of the proliferative block, when full DNA synthesis is restored. Nor can we rule out the possibility that the data represent recombination of vector sequences with cellular DNA perhaps present in an extrachromosomal form. However, the results presented here suggest that regardless of the precise timing of integration, AAV vectors can infect nondividing cells and persist in a stable form that ultimately permits integration of vector sequences. Whether the differences observed in the intensities of hybridizing bands seen in cells arrested in mitosis with aphidicolin compared with actively replicating cells is attributable to the induction of a particular enzyme by aphidicolin which mediates more efficient integration of AAV vector DNA is unclear.

Our results support the premise that the afferent pathway of AAV infection, consisting of particle attachment through virion uncoating with perhaps some degree of second-strand synthesis, is the same within proliferating and nonproliferating cells. With a virion size of 18 to 24 nm (2), AAV virions may be capable of traversing through nuclear pores rather than requiring prior mitosis-mediated nuclear membrane dissolution for entry to the nucleus. Indeed, direct transport through nuclear pores of particles up to 25 nm in diameter, including SV40, another small DNA virus, has been reported (13, 39). Rapid nuclear localization of recombinant AAV capsid proteins has been reported, suggesting the presence of specific mechanisms facilitating nuclear localization (23, 42). Whether AAV-encoded sequences are sufficient for nuclear transport or whether additional cellular proteins are required is unknown. Transgene expression however, would necessitate the import of vector virions to the nucleus prior to transcription.

AAV has a single-stranded linear DNA genome, with strands of each polarity being infectious and packaged separately in equivalent proportions. As transcription requires a double-stranded DNA template, transgene expression in nondividing cells must occur following the conversion of the single-stranded genome to a double-stranded form. Since the experiments reported here were performed at low multiplicities (MOI of $\leq 1$) and transgene expression was observed in a large fraction of cells, it is unlikely that double-stranded templates were generated by the intracellular annealing of input vector genomes of opposite polarities. It is possible that limited DNA synthesis occurs in nondividing cells, which results in the formation of double-stranded templates for transcription. Equivalent levels of expression in dividing and nondividing cells would suggest that all parameters of AAV transduction leading to nuclear localization and second-strand

synthesis occur in FUdR- and aphidicolin-treated and confluent cells at levels equivalent to that in actively dividing cells.

Viral infection followed by integration into nondividing cell populations is not unique to AAV. Recently, HIV-1, a lentivirus, has been shown to infect and integrate into cells that have been growth arrested by gamma irradiation or pretreatment with the DNA synthesis inhibitor aphidicolin or mitomycin, an aziridine antibiotic which covalently binds to and irreversibly cross-links the two DNA strands (30, 31). Integration into growth-arrested cells was dependent on the expression of the virus-encoded, encapsidated integrase gene product (30).

In contrast to our findings with AAV, the proliferative status of the host cell has been shown to play a critical role in the viral replicative cycle following infection with nonlentiviral retroviruses. Infection of growth-arrested fibroblasts with avian or murine retroviruses results in an abortive viral replicative cycle, with disruption at the level of reverse transcription and proviral integration (16, 19). Similarly, active cellular proliferation has been shown to be essential for proviral integration following transduction with retroviral vectors derived from murine leukemia virus (34, 47). Recent studies reveal a requirement for the breakdown of the nuclear membrane, which occurs during mitosis, for nuclear import of the retroviral preintegration complex (41). This differential ability of retroviruses to efficiently transduce only proliferating cells has been elegantly exploited to specifically target rapidly replicating tumor cells within quiescent normal tissue, as in the brain (15). However, this property of retroviral vectors also represents a formidable barrier to efficient gene transfer into a variety of tissues, including peripheral blood T lymphocytes and quiescent totipotent hematopoietic stem cells (38, 40, 50).

This study suggests that safe, efficient, and stable gene transduction is feasible in the absence of DNA synthesis through the use of AAV vectors. The potential for gene transfer into primary hematopoietic cells without the necessity for prior cytokine stimulation suggests that true stem cell gene therapy may be achievable. Indeed, recent studies from our laboratory demonstrated that another AAV-based vector, vCWR:HIVASN (10), was capable of high-efficiency gene transfer into human CD34-enriched bone marrow progenitor cells (12). Thus, the ability of AAV-based vectors to successfully introduce genes into hematopoietic stem cells and other quiescent cell populations provides an exciting new tool for experimental gene transfer studies and potentially for human gene therapy trials.

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