Recombinant Adeno-Associated Virus Mediates a High Level of Gene Transfer but Less Efficient Integration in the K562 Human Hematopoietic Cell Line

PUNAM MALIK,^{1,2} SUSAN A. McQUISTON,³ XIAO-JIN YU,¹ KAREN A. PEPPER,¹ WANDA J. KRALL,¹
GREGORY M. PODSAKOFF,³ GARY J. KURTZMAN,³ AND DONALD B. KOHN^{1*}

*Division of Research Immunology/Bone Marrow Transplantation¹ and Division of Hematology-Oncology,²
Childrens Hospital Los Angeles, University of Southern California School of Medicine,
Los Angeles, and Avigen, Inc., Alameda,³ California*

Received 6 August 1996/Accepted 18 November 1996

We tested the ability of a recombinant adeno-associated virus (rAAV) vector to express and integrate exogenous DNA into human hematopoietic cells in the absence of selection. We developed an rAAV vector, AAV-tNGFR, carrying a truncated rat nerve growth factor receptor (tNGFR) cDNA as a cell surface reporter under the control of the Moloney murine leukemia virus (MoMuLV) long terminal repeat. An analogous MoMuLV-based retroviral vector (L-tNGFR) was used in parallel, and gene transfer and expression in human hematopoietic cells were assessed by flow cytometry and DNA analyses. Following gene transfer into K562 cells with AAV-tNGFR at a multiplicity of infection (MOI) of 1.3 infectious units (IU), 26 to 38% of cells expressed tNGFR on the surface early after transduction, but the proportion of tNGFR expressing cells steadily declined to 3.0 to 3.5% over 1 month of culture. At an MOI of 130 IU, nearly all cells expressed tNGFR immediately posttransduction, but the proportion of cells expressing tNGFR declined to 62% over 2 months of culture. The decline in the proportion of AAV-tNGFR-expressing cells was associated with ongoing losses of vector genomes. In contrast, K562 cells transduced with the retroviral vector L-tNGFR expressed tNGFR in a constant fraction. Integration analyses on clones showed that integration occurred at different sites. Integration frequencies were estimated at about 49% at an MOI of 130 and 2% at an MOI of 1.3. Transduction of primary human CD34⁺ progenitor cells by AAV-tNGFR was less efficient than with K562 cells and showed a declining percentage of cells expressing tNGFR over 2 weeks of culture. Thus, purified rAAV caused very high gene transfer and expression in human hematopoietic cells early after transduction, which steadily declined during cell passage in the absence of selection. Although the efficiency of integration was low, overall integration was markedly improved at a high MOI. While prolonged episomal persistence may be adequate for gene therapy of nondividing cells, a very high MOI or improvements in basic aspects of AAV-based vectors may be necessary to improve integration frequency in the rapidly dividing hematopoietic cell population.

Adeno-associated virus (AAV)-based vectors may have a potential advantage over the currently used retroviral vectors due to their nonpathogenicity, physical stability, and ability to infect dividing as well as nondividing cells, although preferential transduction of dividing cells has been reported (4, 15, 25, 29, 32, 38). Wild-type AAV is a defective parvovirus that requires the help of either adenovirus or herpesvirus for productive cycle or rescue from the host genome. The AAV genome is flanked on both ends with inverted terminal repeats (ITRs) which enclose two open reading frames (ORFs) for the *rep* and *cap* genes (4, 25, 35). In the absence of helper virus, wild-type AAV is known to stably integrate into a specific site at chromosome 19 and remain latent (15, 16, 34). Recombinant AAV (rAAV) vectors consisting of transgenes flanked at both ends with the AAV ITRs can be rescued from a plasmid and packaged into virions in the presence of a helper virus along with a plasmid carrying the *rep* and *cap* ORFs (15, 18, 33, 38).

Several groups have shown that gene transfer into cells by using rAAV vectors occurs efficiently (2, 7, 12, 23, 39, 40, 43). Most reports of high transduction efficiency by rAAV vectors

are based on quantitating gene transfer into cells by DNA PCR for AAV vector sequences and/or evaluation of transgene expression (7, 12, 17, 23, 29, 43). Reports on integration of rAAV are mostly based on vectors encoding a selectable marker which allows for expansion of only the transduced cells (17, 19, 33, 38). AAV vectors are known to persist and express without integrating in nondividing cells of the bronchial epithelium (11). Whether the currently reported high levels of rAAV-mediated gene transfer and expression in hematopoietic cells (12, 17, 30, 37, 39, 43) correlate with integration of the rAAV genome stably into host chromosomes in the absence of selective pressure has not been determined.

We therefore developed an rAAV vector that carried a truncated rat nerve growth factor (NGF) receptor (NGFR) cDNA (tNGFR) as a cell surface reporter under the control of the Moloney murine leukemia virus (MoMuLV) long terminal repeat (LTR) promoter (AAV-tNGFR). An analogous MoMuLV-based retroviral vector, L-tNGFR, was also developed and tested in parallel with the rAAV vector. We quantitatively evaluated gene transfer, expression, and integration from purified rAAV in human hematopoietic cells, without selection. Our results showed that purified rAAV results in very high gene transfer and expression in human hematopoietic cells early after transduction which is dose dependent and declines with cell passage, in the absence of selection. Integration occurs inefficiently into different integration sites and can be

* Corresponding author. Mailing address: Division of Research Immunology/Bone Marrow Transplantation, Mailstop no. 62, 4650 Sunset Blvd., Los Angeles, CA 90027. Phone: (213) 669-2546. Fax: (213) 660-1904. E-mail: dkohn%smtpgate@chla.usc.edu.

FIG. 1. tNGFR-based vectors. (A) The top portion represents a diagram of the full-length rat NGFR cDNA (not to scale). The molecule has an extracellular domain with four cysteine repeats, an NGF-binding site, and an epitope for binding MC192, the monoclonal antibody to tNGFR, the transmembrane domain (TMD), and the cytoplasmic domain (depicted by a bold line and enlarged to show the nucleotide and amino acid sequences and the truncation strategy). The cytoplasmic domain was cleaved with *PvuII* and ligated to the blunt end of the Bluescript plasmid, to generate a truncated cytoplasmic domain followed by a stop codon in frame. The bottom portion shows a cartoon of the resultant tNGFR cDNA. (B) Diagram of the AAV-tNGFR and L-tNGFR vectors with the restriction sites used for Southern blotting. hGH, human growth hormone first intron; SV, SV40 polyadenylation sequence. Black bars below the constructs depict probe fragments used in Southern analyses.

markedly improved by using a very high multiplicity of infection (MOI).

MATERIALS AND METHODS

Truncation of the rat NGFR. The full-length 3.15-kb rat NGFR (p75) cDNA was obtained from Eric Shooter (31) (Stanford University School of Medicine, Stanford, Calif.). The intracytoplasmic portion of the rat NGFR was deleted by digesting the cDNA with *PvuII*, 14 bp downstream of the sequences encoding the transmembrane domain (Fig. 1A), and ligating it with the *SpeI*-digested and blunt end of the Bluescript plasmid [pBS(SK); Stratagene, La Jolla, Calif.] to generate a stop codon in frame. This mutation resulted in a 0.94-kb truncated rat NGFR cDNA (tNGFR) that showed levels of cell surface expression that were similar to those of the full-length rat NGFR cDNA in the LXSN retroviral vector construct (22) when labeled with MC192, the antibody to NGFR (data not shown).

Construction of the retroviral vector L-tNGFR. L-tNGFR vector was based on the G1 vector (18) (provided by Genetic Therapy Inc., Gaithersburg, Md.) and was constructed by cloning the tNGFR cDNA into the *HindIII* site of the G1 plasmid (Fig. 1B). The L-tNGFR plasmid construct was transfected into PA317 packaging cells (20) (obtained from the American Type Culture Collection [ATCC], Rockville, Md.) by using DOTAP transfection reagent (Boehringer Mannheim, Indianapolis, Ind.) along with plasmid pSV2-Neo. The cells were then selected in 0.5 mg of active G418 (Geneticin; Gibco, Grand Island, N.Y.) per ml to enrich for cells with L-tNGFR and then sorted by fluorescence-activated cell sorting (FACS) for tNGFR-expressing (tNGFR⁺) cells (as described below). Viral supernatant from L-tNGFR⁺ PA317 cells was then used to transduce PG13 cells (21) (ATCC) by multiple supernatant exposures. L-tNGFR⁺ PG13 cells were sorted by FACS, and a pool of PG13 cells expressing tNGFR at a high level were used for K562 transductions. Viral titer was determined by infecting HT29 cells, a human colon carcinoma cell line (ATCC), with serial dilutions of viral supernatant (1, 1:5, and 1:10) and quantifying

tNGFR⁺ cells at various dilutions. A bicistronic vector containing both the Neo^r gene and the tNGFR cDNA (L-tNGFR-SN) was concomitantly titered by FACS and by the standard G418 titrating to allow a comparison of Neo titers to that obtained by FACS analysis of the transduced HT29 cells. The titer of the L-tNGFR PG13 cells used in these experiments was estimated at 10^6 infectious units (IU)/ml.

Construction and packaging of AAV-tNGFR. The *NheI-SpeI* segment of the MoMuLV LTR from the LXSN plasmid (22) was bluntly and cloned into the *HpaI* site of a plasmid containing the human growth hormone first intron and the simian virus 40 (SV40) polyadenylation signal (provided by Avigen, Inc., Alameda, Calif.). The tNGFR cDNA was cloned downstream of the MoMuLV LTR. A 2-kb *HindIII* fragment of the bacteriophage lambda was bluntly and inserted upstream of the LTR-tNGFR, into the bluntly *MluI* site, as a stuffer to make the size of the AAV vector similar to that of wild-type AAV (Fig. 1B). A *NotI-NotI* fragment encompassing the λ fragment, the MoMuLV LTR promoter, the human growth hormone first intron, the tNGFR cDNA, and the SV40 polyadenylation signal was then inserted between the ITRs of a pUC-based plasmid containing both the AAV ITRs and the AAV *rep* and *cap* genes outside the ITRs (14). Subconfluent 293 cells were transfected with this plasmid by using calcium phosphate precipitation (14), infected with adenovirus type 2 at an MOI of 2, and incubated at 37°C in 5% CO₂ for 72 h prior to harvest. Cells were lysed in Tris buffer (100 mM Tris, 150 mM NaCl [pH 8.0]) and freeze-thawed three times, and crude-cell lysates were mixed in cesium chloride for isopycnic gradient centrifugation. Recombinant virions were extracted from the resulting gradient by isolating fractions with the average density of approximately 1.38 g/ml, resuspended in HEPES-buffered saline (50 mM HEPES, 150 mM NaCl [pH 7.4]), and heat inactivated at 56°C for 1 h. The heat inactivation eliminated adenovirus at a level of detection of greater than or equal to 100 PFU/ml (14). Using a sensitive PCR assay, we determined the potential wild-type AAV contamination to be approximately 1 in 10^5 vector particles.

The functional titer of AAV-tNGFR was obtained by infecting 293 cells with serial dilutions of AAV-tNGFR vector preparation and determining the percentages of cells positive for surface tNGFR expression by flow cytometry (FACS⁺ cells) 24 h later. The functional titer of the AAV-tNGFR preparation used in all experiments was 1.3×10^8 IU/ml and was used to determine functional MOI. The rAAV encapsidated genome number was quantitated by dot blot hybridization of DNase-treated vector stocks and was found to be 2×10^{13} total genomes/ml.

Transduction of cells with AAV-tNGFR. K562, a human chronic myelogenous leukemia cell line, H9 and CEM, human T-lymphoblastoid cell lines, and U937, a human histiocytic lymphoma cell line, were acquired from ATCC and grown in R10 (RPMI 1640 medium [Gibco-BRL, Bethesda, Md.] with 10% fetal bovine serum [Irvine Scientific, Santa Ana, Calif.], 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml). A total of 10^5 cells were suspended in 0.5 ml of R10 with 1, 10, or 100 μ l of AAV-tNGFR at MOIs of 1.3, 13, and 130. At serial time intervals, half the cells were harvested and washed for FACS and/or DNA analysis.

CD34⁺ cell-enriched progenitor cells were obtained from normal human bone marrow aspirates. Use of these samples was approved by the Committee on Clinical Investigations at Childrens Hospital Los Angeles. Low-density mononuclear cells were obtained by centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) as previously described (27). The cells were then enriched for CD34⁺ cells (typically to >90% purity) by using Mini-MACS columns (Miltenyi Biotech, Auburn, Calif.). CD34⁺ cells (10^5) were plated in 0.5 ml of I-20 (Iscove modified Dulbecco medium [Gibco-BRL] with 20% fetal bovine serum [Irvine Scientific], 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml) on an irradiated (20 Gy) monolayer of 5,000 allogeneic stromal cells in 12-well plates. Stromal cells were derived by culturing the adherent fraction of bone marrow cells as described earlier (27).

CD34⁺ cells were transduced with various MOIs of AAV-tNGFR (1 to 25 μ l of virus, equivalent to MOIs of 1.3 to 32.5 IU/cell) on stroma. After 24 h, the cells were placed into culture in basal bone marrow medium (Iscove modified Dulbecco medium with 30% fetal bovine serum, 1% bovine serum albumin [Sigma], 10^{-4} M 2-mercaptoethanol, 10^{-6} M hydrocortisone, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 2 mM L-glutamine) containing the following cytokines: recombinant human (rH) interleukin-6 (50 U/ml; R&D Systems, Minneapolis, Minn.), rH interleukin-3 (10 ng/ml; Immunex Corp., Seattle, Wash.), and rH mast cell growth factor, a c-Kit ligand (50 ng/ml; Immunex). At days 7 and 14, half of the nonadherent cells were harvested for FACS analysis.

Transduction of cells with the retroviral vector L-tNGFR. A total of 10^5 K562 cells were plated in 0.5 ml of R10 in 12-well plates. Half of the medium was replaced by 1 ml of filtered viral supernatant from L-tNGFR-vector producing PG13 cells either once (experiment 2) or 3 times, 24 h apart over the next 72 h (experiment 3), with the addition of Polybrene to a final concentration of 4 μ g/ml. Control K562 cells were either sham transduced, using R10 medium alone instead of viral supernatants, or transduced with a negative control AAV vector, AAV-LacZ, encoding the bacterial β -galactosidase under the control of the cytomegalovirus immediate-early promoter (14), with all the other transduction conditions remaining the same.

Southern blot analyses. For quantitative Southern blots, genomic DNA was extracted from transduced K562 cells at weeks 2, 3, and 4 after transduction and digested with *EcoRI* and *XhoI*, cutting at sites flanking the tNGFR cDNA. DNA

from a PG13 cell line with a single copy of tNGFR (L-tNGFR-SN clone 9) was also cut with the same restriction enzymes and mixed at various ratios with DNA from nontransduced PG13 cells to generate a standard curve for copy number. Southern blot analyses for proviral integration patterns were done by digesting genomic DNA with *Sst*I, which generates a fragment containing the tNGFR cDNA, the 3' ITR, and adjacent flanking genomic DNA. Digested DNA samples were resolved on 0.7% agarose gels, transferred to nylon filters, and probed with a ³²P-labeled tNGFR cDNA. Filters were washed to a stringency of 2.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and used to expose Kodak AR5 films at -70°C. Relative copy numbers were calculated by scanning blots for beta emission on Betascope (Betagen Corp., Waltham, Mass.). Integrity of the MoMuLV LTR-tNGFR expression cassette and the methylation status of the MoMuLV LTR promoter were assessed by digesting genomic DNA with *Afl*II and *Xho*I, which cut the MoMuLV LTR-tNGFR expression cassette from the AAV-tNGFR vector. Portions of the same DNA digested with *Afl*II-*Xho*I were further digested with the methylation-sensitive restriction enzyme *Sma*I, which cuts once within the MoMuLV LTR at position +30 relative to the transcriptional initiation site. Digested genomic DNA was run and blotted as described above and probed with a ³²P-labeled MoMuLV LTR fragment which corresponded to a region of the MoMuLV LTR upstream of the *Sma*I site.

Antibody staining and FACS analysis. Cells were harvested, washed twice with RPMI 1640, and suspended at a concentration of 10⁷ cells/ml. Thereafter, antibody staining was done at 4°C. One million cells were incubated with 6 µl of human immunoglobulin (10 mg/ml) for 30 minutes to block nonspecific antibody binding. Five microliters of the antibody to rat NGFR, MC192 (100 µg/ml; Oncogene Science, Uniondale, N.Y.), was added per 10⁶ cells for 30 min. Cells were then washed two times with phosphate-buffered saline to remove excess antibody, counterstained with phycoerythrin-labeled goat anti-mouse antibody (Caltag, San Francisco, Calif.), and fixed in 1% paraformaldehyde. Samples were subjected to flow cytometry on a FACScan (Becton Dickinson, San Jose, Calif.) or sorted on a FACS Vantage flow cytometer (Becton Dickinson). Either sham-transduced cells or cells transduced with the AAV-LacZ vector were concomitantly stained with MC192 as negative controls. Negative control cells were used to set gates such that more than 99% of sham-transduced cells were excluded for tNGFR expression.

PCR analyses. PCR was performed on genomic DNA from K562 cells to amplify a 560-bp product within the tNGFR cDNA. Oligonucleotide primers were TGAACCCCTGCCTGACAATG (sense) and AGCAGCCAAGATGGA GCAATAGAC (antisense). PCR conditions were as follows: 94°C for 5 min, 58°C for 2 min, and 72°C for 2 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min. PCR products were resolved on 1.2% agarose gels and visualized with ethidium bromide, using UV illumination.

RESULTS

rAAV and retroviral vectors expressing the tNGFR surface antigen reporter. As a reporter gene which could be measured by FACS analysis, we generated a truncated rat nerve growth factor cDNA (tNGFR [Fig. 1A]). Analogous rAAV and retroviral vectors carrying the tNGFR were produced (Fig. 1B). The MoMuLV LTR was used as the promoter controlling transcription of the tNGFR cDNA in both the rAAV and the retroviral vector to maintain uniformity.

AAV-tNGFR expression in different cell types. The ability of the rAAV vector to express the tNGFR reporter was tested in a variety of human hematopoietic and lymphoid cell lines. K562, CEM, H9, and U937 cells were infected with AAV-tNGFR (MOI of 13) under identical transduction conditions (proportion of cells, transduction volume, lot of virus, and MOI), and expression of tNGFR studied by FACS at days 2, 4, and 7 posttransduction (Fig. 2). The proportion of cells showing tNGFR surface expression varied widely in these cell types, with the highest expression (in 96% of the cells) seen in K562 cells. Percentages of cells showing tNGFR surface expression declined by 30 to 50% in all cell types by day 7.

Temporal course of AAV-tNGFR expression without selective pressure. The stability of gene transfer and expression by rAAV was then studied in K562 cells over 2 months, in the absence of selection. K562 cells were transduced with an MOI of 1.3, 13, or 130 (experiment 1). Half of the transduced cells were harvested periodically and analyzed by FACS for tNGFR expression (Fig. 3). The proportion of cells showing AAV-tNGFR expression at day 4 following transduction progressively increased from 34.8 to 94% as the MOI increased from

FIG. 2. AAV-tNGFR expression in different hematopoietic cell types. K562, H9, CEM, and U937 cells were infected with AAV-tNGFR at an MOI of 13 IU under identical transduction conditions. Half the cells were harvested at days 2, 4, and 7, labeled with MC192, and analyzed by FACS for percentage of tNGFR-positive cells, plotted on the y axis. K562 cells transduced by AAV-LacZ were used as a negative control.

1.3 to 130. There was a progressive decline in the proportion of cells expressing tNGFR over 8 weeks. At an MOI of 130, the decline was less pronounced and 61.9% of cells expressed tNGFR at 8 weeks.

Declining NGFR expression was due to loss of AAV genomes. To ascertain the cause of decline in the number of cells expressing AAV-tNGFR, we compared transduction of AAV-tNGFR to that of an analogous retroviral vector, L-tNGFR, by the schema shown in Fig. 4A. K562 cells were transduced with AAV-tNGFR at an MOI of 1.3 or with the retroviral vector L-tNGFR. Sham- or AAV-LacZ-transduced cells served as the negative control. Half of the transduced cells were harvested weekly for 4 weeks and analyzed both by flow cytometry, for tNGFR expression, and by quantitative Southern blotting, for rAAV- and retrovirus-mediated gene transfer.

Table 1 depicts the results of one representative experiment (experiment 3). In K562 cells transduced with AAV-tNGFR, the proportion of cells expressing tNGFR progressively declined over 4 weeks, from 26% at 1 week to 3.5% at 4 weeks. In contrast, in K562 cells transduced with the retroviral vector L-tNGFR, the proportion of cells expressing the transgene was relatively constant. Figure 4B shows the FACS analyses of the K562 cells in this experiment at 4 weeks posttransduction; only 3.5% AAV-tNGFR-transduced cells expressed tNGFR, while

FIG. 3. Temporal analysis of tNGFR expression with increasing MOI of AAV-tNGFR. K562 cells were transduced with AAV-tNGFR at MOIs of 1.3, 13, and 130. Sham-transduced (MOI of 0) K562 cells served as the negative controls. Some of the transduced cells were harvested periodically and were analyzed for tNGFR expression by FACS. The bar diagram depicts the percentages of the K562 cells that were expressing tNGFR on the cell surface over 8 weeks of culture when stained with the antibody to NGFR, MC192, and analyzed by FACS.

FIG. 4. Temporal analysis of tNGFR expression by rAAV and an analogous retroviral vector without selective pressure. (A) Experimental design of the integration experiments performed with K562 cells that were transduced with AAV-tNGFR and an analogous retroviral vector, L-tNGFR. Sham-transduced or AAV-LacZ-transduced K562 cells served as the negative controls. Half of the transduced cells were harvested weekly and were analyzed for tNGFR expression by FACS and for vector copy number/cell by quantitative Southern blots (Qtt. S.B.). After 4 or 8 weeks, tNGFR+ cells were sorted as single cells, and clones were expanded over the next 2 to 3 months. Clones were screened for the presence of the tNGFR cDNA by PCR. PCR+ clones were analyzed for expression by FACS and for integration pattern by Southern blotting. AAV-tNGFR K562 clones with integrated proviruses that did not express tNGFR (FACS-) were further analyzed by Southern blots for proviral integrity and methylation status of the MoMuLV promoter at the *Sma*I site. (B) FACS analyses of K562 cells in one experiment transduced with the negative control vector AAV-LacZ (left), with the retroviral vector, L-tNGFR (middle), and with AAV-tNGFR (right) 4 weeks posttransduction. These cells were labeled with MC192 (along the y axes) as described in Materials and Methods. The tNGFR+ cells were determined by setting markers or quadrants above the tNGFR expression of the negative control cells, resulting in exclusion of about 99.5% of the negative control cells (left). Cells depicted in the R2 gate were FACS sorted as single cells into 96-well plates, using an automated cell deposition unit. (C) Quantitative Southern blot analysis of the stability of gene insertion in K562 cells, comparing AAV vector to retroviral vector. Quantitative Southern blot analysis was performed on pools of K562 cells transduced with AAV-tNGFR, L-tNGFR, or the negative control AAV-LacZ and harvested at weeks 2, 3, and 4 (Table 1). Genomic DNA was extracted and digested with *Xho*I and *Ssr*I, cutting at sites flanking the tNGFR cDNA. DNA from a cell line with a single copy of the tNGFR was also cut with the same restriction enzymes to generate a standard (Std) curve for copy number. The blot was probed with the ³²P-labeled tNGFR cDNA. Positions of molecular weight markers (M.W.) are indicated in kilobases.

64% of the L-tNGFR-transduced cells continued to express the transgene.

Figure 4C shows the quantitative Southern blot analysis of AAV-LacZ-, AAV-tNGFR-, and L-tNGFR-transduced K562 cells in the experiment described above. Genomic DNA extracted at 2, 3, and 4 weeks was digested by cutting at sites flanking the tNGFR cDNA. K562 cells transduced by the retroviral vector L-tNGFR had close to one copy per cell at weeks 2, 3, and 4, corresponding to 67, 79, and 64% tNGFR expression, respectively. In contrast, AAV-tNGFR-infected cells showed a large number (>67) of rAAV genomes per cell at week 2, which sharply declined by weeks 3 and 4 to 3.6 and 0.15 copies/cell, respectively. During this time, the percentages of cells expressing tNGFR were 21, 7.7, and 3.5% (Table 1).

Expression and integration analyses of AAV-tNGFR clones. K562 cells expressing tNGFR on their surface were sorted as single cells (sort gates of the experiment described above, R2,

shown in Fig. 4B) 8 and 4 weeks posttransduction. Clones were expanded over the next 2 to 4 months, and 75 individual clones were analyzed for AAV-tNGFR integration as well as for surface expression of tNGFR. PCR was performed to screen individual clones for the presence of tNGFR. Table 2 summarizes the PCR, FACS, and integration analyses from all clones tested.

At the higher MOI of 130, 3 of 24 (12%) of the clones sorted as expressing tNGFR at 8 weeks were no longer positive for presence of vector DNA by PCR at 5 to 6 months (i.e., were PCR-); the remaining 21 of 24 (88%) of the clones were vector positive by PCR (i.e., were PCR+) and also expressed tNGFR on the surface (Table 2).

At an MOI of 1.3, 16 of 51 (31.4%) of the clones that were sorted as expressing tNGFR at 4 weeks were no longer positive for tNGFR DNA by PCR at 3 to 4 months (Table 2). The remaining 35 clones that were vector positive by PCR (68.6%)

TABLE 1. Proportion of K562 cells transduced with AAV-tNGFR and the analogous retroviral vector L-tNGFR expressing tNGFR on the cell surface over 4 weeks of culture

Days	% tNGFR+ cells ^a		
	AAV-LacZ	AAV-tNGFR ^b	L-tNGFR ^c
7	0.2	26.0	73.0
14	0.3	20.8	66.7
21	0.0	7.7	79.3
28	0.0	3.5	63.8

^a Expression of tNGFR was analyzed by labeling cells with the MC192 antibody and analyzing the percentages of tNGFR cells by FACS as described in Materials and Methods.

^b MOI of AAV-tNGFR was 1.3 IU.

^c MOI of L-tNGFR was estimated at 30 IU.

were then analyzed by FACS for expression of the transgene. Twenty-one of 35 (60%) of the PCR+ clones expressed tNGFR on the cell surface, while 14 of 35 (40%) of the PCR+ clones did not express tNGFR on the surface.

Integration Southern blots were performed by digesting genomic DNA from the clones with *Sst*I, an enzyme that would cut within the provirus, releasing a fragment containing the 1.2-kb tNGFR cDNA and 3' ITR along with flanking chromosomal DNA (Fig. 1B). Figure 5 shows a representative Southern blot analysis on clones derived from experiment 3. All bands detected were larger than 1.2 kb when hybridized with the tNGFR cDNA probe, consistent with their origin from the vector-genomic DNA junction. Even in the event of AAV-tNGFR forming multiple episomal concatemers, the tNGFR probe would detect constant-size bands of 1.8 kb (head-tail) or 2.4 kb (tail-tail), assuming no ITR deletions. As shown in Fig. 5, all clones that had integrated vectors showed different band sizes, consistent with different sites of chromosomal integration.

Overall, at an MOI of 130, 19 of 24 clones (79%) showed integrated proviruses. These clones were derived from the 62% of the total cell population which was expressing tNGFR (at 2 months after transduction). Hence, the integration frequency of AAV-tNGFR at an MOI of 130 was estimated to be 49%.

TABLE 2. PCR, FACS, and integration analyses of AAV-tNGFR K562 single-cell-derived clones sorted on the basis of expression of tNGFR

Expt	Day of sort ^a	MOI (IU)	Total no. of clones analyzed	Proportion of FACS-sorted clones:				
				PCR- ^b	PCR+	PCR+/FACS- ^c	PCR+/FACS+	FACS+/SB+ ^d
1	56	130	24	3/24	21/24	0/21	21/21	19/21
2	28	1.3	19	6/19	13/19	7/13	6/13	6/6
3	28	1.3	32	10/32	22/32	7/22	15/22	15/15

^a K562 cells that were positive for AAV-tNGFR expression by FACS were sorted as single cells by using a FACS Vantage automated cell deposition unit, and clones were expanded over 3 to 4 months before PCR, FACS, and Southern blot analyses as described in Materials and Methods.

^b DNAs from clones were initially screened by the PCR assay, as described in Materials and Methods, to detect the presence of the tNGFR cDNA. PCR- clones showed no detectable PCR product, and PCR+ clones showed a distinct product by ethidium bromide staining.

^c PCR+ clones were analyzed by flow cytometry by staining with the antibody to NGFR, MC192, as described in Materials and Methods. Cells from FACS- clones showed no tNGFR surface expression, and nearly all cells from FACS+ clones expressed NGFR on the surface.

^d Integration Southern blot analyses were performed by digesting genomic DNA from each clone with *Sst*I and probing with the tNGFR cDNA as described in Materials and Methods. SB+ clones showed one to three integrants.

Two of 21 clones did not show integrants and hence may have been expressing tNGFR from unintegrated virus, even at 5 to 6 months posttransduction. At an MOI of 1.3, about two-thirds of the clones showed integrated proviruses. These clones were derived from the 3 to 3.5% of the total cell population which was expressing tNGFR at the time of FACS sorting (1 month after transduction). Hence, the integration frequency of AAV-tNGFR in the absence of selection was estimated at approximately 2%.

The quantitative levels of tNGFR surface expression by AAV-tNGFR and L-tNGFR in clones that had single vector integrants by Southern blot were comparable.

DNA analysis of vector-positive nonexpressing clones. Four K562 clones with integrated AAV-tNGFR proviruses but no surface expression of tNGFR were also analyzed by Southern blot to determine the integrity of the expression cassette and to assess methylation of the MoMuLV LTR promoter in the AAV backbone (Fig. 6). The *Afl*II-*Xho*I digests of all AAV-tNGFR clones (lanes marked - in Fig. 6) showed that the LTR-tNGFR expression cassette was of the predicted 2-kb size in all clones expressing tNGFR as well as the nonexpressing clones. One clone showed an additional higher-molecular-weight vector band (lane 9).

Further digestion of the same DNA with *Sma*I (lanes marked + in Fig. 6) resulted in complete digestion at the *Sma*I site in all of the AAV-tNGFR-expressing and three of four nonexpressing clones. One clone showed persistence of the 2-kb band (lane 10), which suggests an incomplete *Sma*I digestion due to partial methylation of the MoMuLV LTR at the *Sma*I site. Alternatively, this 2-kb band may represent the *Sma*I-digested product of the additional rearranged vector band seen in this clone (lane 9).

AAV-tNGFR expression in primary human CD34⁺ cells. CD34⁺ cells required much higher infectious units of AAV vector for any observable level of expression compared to K562 cells (Table 3). Minimal tNGFR expression from CD34⁺ cells was detected by FACS following transduction by AAV-tNGFR at an MOI of <6.5 (data not shown). Increasing the MOI of AAV-tNGFR increased the percentage of cells expressing tNGFR. Eight to 15.5% of the cells expressed tNGFR at day 7, but the percentages of cells expressing tNGFR on the surface dropped to minimally detectable levels by 2 weeks. Increasing the MOI to 32.5 resulted in cellular toxicity, manifested as lower viable cell counts and reduced numbers of CFU. The low expression from CD34⁺ cells (in the range of 2 to 5% of tNGFR+ cells) was reproducible on repeated transductions of CD34⁺ cells from either umbilical cord blood or bone marrow (data not shown).

DISCUSSION

In this study, we report gene transfer, expression, and integration of a rAAV vector in human hematopoietic cells, in the absence of selection. We used the rat tNGFR cDNA as a cell surface reporter to allow expression studies by FACS and study of the integration pattern in individual FACS-sorted clones by Southern blot analyses. K562 cells were transduced with AAV-tNGFR and were studied over a period of 3 to 6 months without selective pressure.

Gene transfer into K562 cells was very efficient. The declining pattern of AAV-tNGFR expression in K562 cells was associated with the loss of vector genomes with cell passage, observed in Southern blot analyses of pools of cells. Loss of vector genome was probably not due to the elimination of cells from a toxic effect of the rat tNGFR cDNA, since K562 cells transduced in parallel with an analogous retroviral vector, L-

FIG. 5. Integration and FACS analysis of AAV-tNGFR clones sorted as tNGFR+ at 1 month posttransduction. Integration Southern blot analyses were performed on all AAV-tNGFR clones that were PCR+/FACS+ and some clones that were PCR+/FACS- as described in Materials and Methods. DNA from a K562 clone that was PCR-/FACS- and from nontransduced K562 cells served as controls for the endogenous human NGFR gene (marked by arrows on the left).

tNGFR, showed a constant vector copy number/cell over 4 weeks of culture. tNGFR expression should not result in alteration in the function of the transduced cells, since an identical truncation of the cytoplasmic domain of human NGFR cDNA results in loss of NGF-induced tyrosine phosphorylation (3)

FIG. 6. Southern blot analysis to test the integrity of the LTR-tNGFR expression cassette and methylation of the LTR promoter in K562 clones not expressing from integrated AAV-tNGFR. Genomic DNAs from the four K562 clones containing integrated AAV-tNGFR proviruses which did not express tNGFR (clones 1 and 8 from experiment 2 and clones 9 and 28 from experiment 3) were digested with *Afl*II and *Xho*I, enzymes flanking the 2-kb LTR-tNGFR expression cassette (lanes 7, 9, 11, and 13), and a portion of these DNAs was further digested with *Sma*I, which cuts once in the LTR (lanes 8, 10, 12, and 14), resulting in a 0.5-kb LTR fragment when probed with a ³²P-labeled MoMuLV LTR fragment. DNA from AAV-tNGFR-expressing clones with a single integrant (lanes 3 to 6) were also digested similarly as positive controls for size and for absence of methylation. DNA from nontransduced K562 cells (lanes 1 and 2) served as a negative control. Complete *Sma*I digestion of each sample was checked by concordant digestion of adenovirus DNA in a portion of the genomic DNA-*Sma*I-digestion mix.

and the human and rat NGFRs are highly conserved in this region (41).

The extremely high AAV-tNGFR copy number/cell seen initially in the quantitative Southern blot probably reflects the high genome number per infectious rAAV particle in the vector preparation used. tNGFR expression in the same K562 cells was more reflective of the functional MOI of 1.3. Hence, reports of high-efficiency transduction with rAAV that utilize DNA PCR should be interpreted cautiously, since DNA PCR will clearly be positive for a high copy number due to the presence of rAAV genomes which may not correlate with transgene expression. This difference between vector copy number/cell and expression was not observed in K562 cells transduced with the analogous retroviral vector, L-tNGFR.

Loss of AAV vector genomes continued even after 1 month in culture. At an MOI of 1.3, one-third of the cells that were expressing tNGFR at the time of FACS sorting (1 month after transduction) were no longer positive for vector DNA by 3 to 4 months. At an MOI of 130, 12% of the cells that were expressing tNGFR at the time of FACS sorting (2 months after transduction) were no longer positive for vector DNA by 5 to 6 months. Two clones (8%) that expressed the transgene 5 to 6 months after transduction lacked detectable integrated vector sequences and apparently expressed tNGFR from persistent nonintegrated vector. The decline in rAAV genomes occurred over a more prolonged time than that seen in standard transient transfections of cells using plasmid DNA. Prolonged episomal persistence of rAAV in the relatively nondividing

TABLE 3. AAV-tNGFR expression in human CD34⁺ cells

Days	% tNGFR+ cells ^a after infection at MOI of:			
	0	6.5	13	32.5
7	0	8.3	9.0	15.5
14	0	1.6	2.0	0.0

^a Expression of tNGFR was analyzed by labeling cells with the MC192 antibody and analyzing the percentages of tNGFR+ cells by FACS as described in Materials and Methods.

cells of bronchial epithelium has been reported by Flotte et al. (11). Recently, Philip et al. reported that the presence of AAV ITRs in plasmids can confer a prolonged persistence of transfected plasmid DNA in cells compared to transfection mediated by standard plasmids lacking AAV ITRs (28). Previous studies that have reported efficient gene transfer of rAAV into nonhematopoietic cells (10, 17, 29, 36, 38), murine hematopoietic cells (7, 40), and human hematopoietic cells (7, 12, 17, 24, 30, 37, 39, 40, 43) have not established whether the high-efficiency gene transfer is coincident with stable integration. Our data are consistent with very efficient gene transfer followed by a prolonged but declining episomal presence of rAAV in the majority of cells in the rapidly dividing hematopoietic cell population. Recently similar results were reported by Bertran et al. for HeLa epithelial cells (5). Our study confirms these results for hematopoietic cells. Additionally, analyses of integration frequency at different MOIs show improved integration rates by the use of a very high MOI.

Integration analyses showed that overall, integration occurred inefficiently in the absence of selective pressure, occurring in approximately 2% of the cells transduced at an MOI of 1.3. However, a 100-fold-higher MOI (130 IU) resulted in integration in approximately 49% of the cells, improving integration frequency by 25-fold. The mechanism underlying the higher extent of stable transduction at a 100-fold-higher MOI is not known. Potentially, the higher number of input viral genomes may simply increase the probability of one copy succeeding in integrating into the cellular chromosome; alternatively, some auxiliary protein associated with the AAV virion in trace amounts, such as the AAV Rep or the adenovirus E4 ORF6 protein (9, 26), may augment integration.

Site-specific integration of wild-type AAV into chromosome 19 has been well documented (16, 34). Reports on integration of rAAV vectors into cells are mainly based on vectors encoding the neomycin resistance gene, and most of these suggest that integration of rAAV is not site specific and frequently occurs in tandem repeats (8, 16, 23, 30, 33, 36, 38, 39). Reports on integration of rAAV into hematopoietic cells without selection are very few and are based on detectable vector sequences 3 to 4 months after transduction and not on demonstration of vector-genomic DNA junctional fragments (37, 40). Our data suggest non-site-specific integration of purified rAAV, based on detection of different sizes of vector-genomic DNA fragments among different clones.

The MoMuLV LTR-tNGFR expression cassette of AAV-tNGFR was intact in all four transcriptionally silent clones with integrated proviruses. One of these clones showed an additional, rearranged vector band. Thus, transcriptional inactivity was not due solely to rearrangements of the expression portion of the proviruses. We have previously reported that a lack of expression by MoMuLV-based retroviral vectors was associated with methylation of the MoMuLV LTR at the *Sma*I site in a murine gene transfer/bone marrow transplantation model (6). In the studies reported here, at least three of four clones which failed to express from integrated AAV-tNGFR did not show methylation at the *Sma*I site in the MoMuLV LTR, used to express the tNGFR cDNA. Thus, the reasons some clones failed to express from intact proviruses are not known.

Primary human CD34⁺ cells showed lower percentages of cells expressing tNGFR compared to the K562 cells transduced at a comparable MOI. Attempts to increase the MOI of AAV-tNGFR (>13) resulted in toxicity to the CD34⁺ cells. The pattern of decline in expression in CD34⁺ cells was similar to that in K562 cells and the other hematopoietic cell lines. Low percentages of CD34⁺ cells expressing AAV-tNGFR were probably not due to low levels of expression of the tNGFR

transgene in human CD34⁺ cells, as we have observed good expression of tNGFR from a retroviral vector in CD34⁺ cells. Halbert et al. have reported lower transductions of primary cells compared to immortalized cells with rAAV vectors (13).

Higher transductions of primary human CD34⁺ cells by using rAAV have been reported by some investigators (12, 23, 40, 43). Many of the initial reports of high CD34⁺ cell transduction efficiency with rAAV are based on DNA PCR, which may reflect only efficient entry of rAAV particles (12, 23, 43). Studies on transgene expression in CD34⁺ cells are often based on reverse transcription-PCR (12, 23), which may be much more sensitive than detection of tNGFR surface expression. High CD34⁺ cell transduction with rAAV, based on selection of CFU in G418, has been shown (43). Walsh et al. (40) have reported in vitro functional correction of the Fanconi anemia phenotype by transduction of CD34⁺ cells with rAAV-based vectors. Their success may be due to a natural selective advantage of corrected cells when grown in mitomycin-containing media. Increased AAV transduction in the presence of active DNA repair in hematopoietic cells from Fanconi anemia patients may also play a role, as DNA-damaging agents have been reported to increase transduction with rAAV, probably due to induction of active DNA repair (1, 9). Alternatively, our results of low expression in CD34⁺ cells from rAAV may also be attributed to variations in packaging and purification of the rAAV virus.

In conclusion, we report the temporal course of a purified rAAV vector infection in human hematopoietic cells, in the absence of selection. Our data show that rAAV mediates high gene transfer and expression that steadily declines with cell passage. The efficiency of stable integration is low and may be improved by using a very high MOI. Although the prolonged episomal persistence of rAAV can be exploited for gene therapy of nondividing cells, better understanding of AAV vector biology is needed to design and package AAV vectors for hematopoietic cells, where higher levels of integration into quiescent hematopoietic stem cells are necessary for effective gene therapy applications.

ACKNOWLEDGMENTS

We thank E. Monika Smogorzewska and Felix Burotto for expert technical assistance in cell sorting on the FACS Vantage flow cytometer.

This study was supported by research grants from the Department of Health and Human Services, NIH, NIDDK (DK 42694-01A1), and the Concern II Foundation.

REFERENCES

- Alexander, I. E., D. W. Russell, and D. Miller. 1994. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J. Virol.* **68**:8282–8287.
- Antoni, B. A., A. B. Rabson, I. L. Miller, J. P. Trempe, N. Chejanovsky, and B. J. Carter. 1991. Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. *J. Virol.* **65**:396–404.
- Berg, M. M., D. W. Sternberg, B. L. Hempstead, and M. V. Chao. 1991. The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* **88**:7106–7110.
- Berns, K. L., and R. A. Bohenzky. 1987. Adeno-associated viruses: an update. *Adv. Virus Res.* **32**:243–307.
- Bertran, J., J. L. Miller, Y. Yang, A. Fenimore-Justman, F. Rueda, E. F. Vanin, and A. W. Neinhuis. 1996. Recombinant adeno-associated virus-mediated high efficiency transient expression of the murine cationic amino acid transporter (ecotropic retroviral receptor) permits stable transduction of human HeLa cells by ecotropic retroviral vectors. *J. Virol.* **70**:6759–6766.
- Challita, P., and D. Kohn. 1994. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc. Natl. Acad. Sci. USA* **91**:2567–2571.
- Chatterjee, S., P. R. Johnson, and K. K. Wong, Jr. 1992. Dual-target inhibition of HIV-1 in vitro by means of an adeno-associated virus antisense vector. *Science* **258**:1485–1488.

8. **Einerhand, M. P., M. Antoniou, S. Zolotukhin, N. Muzyczka, K. I. Berns, F. Grosfeld, and D. Valerio.** 1995. Regulated high-level human beta-globin gene expression in erythroid cells following recombinant adeno-associated virus-mediated gene transfer. *Gene Ther.* **2**:336-343.
9. **Ferrari, F. K., T. Samulski, T. Shenk, and R. J. Samulski.** 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J. Virol.* **70**:3227-3234.
10. **Flotte, T. R., S. A. Afione, R. Solow, M. L. Drumm, D. Markakis, W. B. Guggino, P. L. Zeitlin, and B. J. Carter.** 1993. Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. *J. Biol. Chem.* **268**:3781-3790.
11. **Flotte, T. R., S. A. Afione, and P. L. Zeitlin.** 1994. Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am. J. Respir. Cell Mol. Biol.* **11**:517-521.
12. **Goodman, S., X. Xiao, R. E. Donahue, A. Moulton, J. Miller, C. Walsh, N. S. Young, R. J. Samulski, and A. W. Nienhuis.** 1994. Recombinant adeno-associated virus-mediated gene transfer into hematopoietic progenitor cells. *Blood* **84**:1492-1500. (Erratum, **85**:862, 1995.)
13. **Halbert, C. L., I. E. Alexander, G. M. Wolgamot, and A. D. Miller.** 1995. Adeno-associated virus vectors transduce primary cells much less efficiently than immortalized cells. *J. Virol.* **69**:1473-1479.
14. **Kessler, P. D., G. M. Podsakoff, X. Chen, S. A. McQuiston, P. C. Colosi, L. A. Matelis, G. J. Kurtzman, and B. J. Byrne.** 1996. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA* **93**:14082-14087.
15. **Kotin, R. M.** 1994. Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum. Gene Ther.* **5**:793-801.
16. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. Zhu, L. Hunter, K. A. Laughlin, S. K. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87**:2211-2215.
17. **Lebkowski, J. S., M. M. McNally, T. B. Okarma, and L. B. Lerch.** 1988. Adeno-associated virus: a vector system for efficient introduction and integration of DNA into a variety of mammalian cell types. *Mol. Cell. Biol.* **8**:3988-3996.
18. **McLachlin, J. R., N. Mittereder, M. B. Daucher, M. Kadan, and M. A. Eglitis.** 1993. Factors affecting retroviral vector function and structural integrity. *Virology* **195**:1-5.
19. **McLaughlin, S. K., P. Collis, P. L. Hermonat, and N. Muzyczka.** 1988. Adeno-associated virus general transduction vectors: analysis of proviral structures. *J. Virol.* **62**:1963-1973.
20. **Miller, A. D., and C. Buttimore.** 1986. Redesign of retrovirus packaging lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895-2902.
21. **Miller, A. D., J. V. Garcia, N. V. Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden.** 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65**:2220-2224.
22. **Miller, D., and G. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7**:980-986.
23. **Miller, J. L., R. E. Donahue, S. E. Sellers, R. J. Samulski, N. S. Young, and A. W. Nienhuis.** 1993. Recombinant adeno-associated virus (rAAV)-mediated expression of a human gamma-globin gene in human progenitor-derived erythroid cells. *Proc. Natl. Acad. Sci. USA* **91**:10183-10187.
24. **Miller, J. L., C. E. Walsh, P. A. Ney, R. J. Samulski, and A. W. Nienhuis.** 1993. Single-copy transduction and expressions of human gamma-globin in K562 erythroleukemia cells using recombinant adeno-associated virus vectors: the effect of mutations in NF-E2 and GATA-1 binding motifs within the hypersensitivity site 2 enhancer. *Blood* **82**:1900-1906.
25. **Muzyczka, N.** 1992. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* **158**:97-129.
26. **Natsoulis, G., R. Suroski, S. Godwin, S. McQuiston, and G. Kurtzman.** 1995. A system for specifically targeting genes into chromosome 19. *Blood* **86**(Suppl. 1):236a.
27. **Nolta, J. A., X. Yu, I. Bahner, and D. Kohn.** 1992. Retroviral mediated transfer of the human glucocerebrosidase gene into cultured Gaucher bone marrow. *J. Clin. Invest.* **90**:343-348.
28. **Philip, R., E. Brunette, L. Kilinski, D. Murugesu, M. A. McNally, K. Ucar, J. Rosenblatt, T. B. Okarma, and J. S. Lebkowski.** 1994. Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol. Cell. Biol.* **14**:2411-2418.
29. **Podsakoff, G., K. K. Wong, Jr., and S. Chatterjee.** 1994. Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. *J. Virol.* **68**:5656-5666.
30. **Ponnazhagan, S., M. L. Nallari, and A. Srivastava.** 1994. Suppression of human alpha-globin gene expression mediated by the recombinant adeno-associated virus 2-based antisense vectors. *J. Exp. Med.* **179**:733-738.
31. **Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter.** 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* **325**:593-597.
32. **Russell, D. W., A. D. Miller, and I. E. Alexander.** 1994. Adeno-associated virus vectors preferentially transduce cells in S phase. *Proc. Natl. Acad. Sci. USA* **91**:8915-8919.
33. **Samulski, R. J., L. Chang, and T. Shenk.** 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* **63**:3822-3828.
34. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* **10**:3941-3950.
35. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* **45**:555-564.
36. **Sun, X., B. R. Murphy, Q. Li, S. Gullapalli, J. Mackins, H. N. Jayaram, A. Srivastava, and A. C. Antony.** 1995. Transduction of folate receptor cDNA into cervical carcinoma cells using recombinant adeno-associated viruses delays cell proliferation in vitro and in vivo. *J. Clin. Invest.* **96**:1535-1547.
37. **Thrasher, A. J., M. de Alwis, C. M. Casimir, C. Kinnon, K. Page, J. S. Lebkowski, A. W. Segal, and R. J. Levinsky.** 1995. Functional reconstitution of the NADPH-oxidase by adeno-associated virus gene transfer. *Blood* **86**:761-765.
38. **Tratschin, J. D., I. L. Miller, M. G. Smith, and B. J. Carter.** 1985. Adeno-associated virus vector for high-frequency integration, expression, and rescue of genes in mammalian cells. *Mol. Cell. Biol.* **5**:3251-3260.
39. **Walsh, C. E., J. M. Liu, X. Xiao, N. S. Young, A. W. Nienhuis, and R. J. Samulski.** 1992. Regulated high level expression of a human gamma-globin gene introduced into erythroid cells by an adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* **89**:7257-7261.
40. **Walsh, C. E., A. W. Nienhuis, R. J. Samulski, M. G. Brown, J. L. Miller, N. S. Young, and J. M. Liu.** 1994. Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated virus vector. *J. Clin. Invest.* **94**:1440-1448.
41. **Welcher, A. A., C. M. Bitler, M. J. Radeke, and E. M. Shooter.** 1991. Nerve growth factor binding domain of the nerve growth factor receptor. *Proc. Natl. Acad. Sci. USA* **88**:159-163.
42. **Zhou, S. Z., H. E. Broxmeyer, S. Cooper, M. A. Harrington, and A. Srivastava.** 1993. Adeno-associated virus 2-mediated gene transfer in murine hematopoietic progenitor cells. *Exp. Hematol.* **21**:928-933.
43. **Zhou, S. Z., S. Cooper, L. Y. Kang, L. Ruggieri, S. Heimfeld, A. Srivastava, and H. E. Broxmeyer.** 1994. Adeno-associated virus 2-mediated high efficiency gene transfer into immature and mature subsets of hematopoietic progenitor cells in human umbilical cord blood. *J. Exp. Med.* **179**:1867-1875.