Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors

SIHONG SONG*, MICHAEL MORGAN*, TAMIR ELLIS†, AMY POIRIER*, KYE CHESNUT*, JIANMING WANG*, MARK BRANTLY‡, NICHOLAS MUZYCZKA*, BARRY J. BYRNE*, MARK ATKINSON†, AND TERENCE R. FLOTTE*§

*Gene Therapy Center, University of Florida, Box 100266, Gainesville, FL 32610; †Department of Pathology, University of Florida, Gainesville, FL 32610; and ‡Pulmonary Branch, National Heart, Lung and Blood Institute, Bethesda, MD 20892

Communicated by Kenneth I. Berns, University of Florida College of Medicine, Gainesville, FL, October 5, 1998 (received for review May 26, 1998)

ABSTRACT Recombinant adeno-associated virus (AAV) vectors have been used to transduce murine skeletal muscle as a platform for secretion of therapeutic proteins. The utility of this approach for treating alpha-1-antitrypsin (AAT) deficiency was tested in murine myocytes *in vitro* and *in vivo*. AAV vectors expressing the human AAT gene from either the cytomegalovirus (CMV) promoter (AAV-C-AT) or the human elongation factor 1- α promoter (AAV-E-AT) were examined. *In vitro* in C2C12 murine myoblasts, the expression levels in transient transfections were similar between the two vectors. One month after transduction, however, the human elongation factor 1 promoter mediated 10-fold higher stable human AAT expression than the CMV promoter. *In vivo* transduction was performed by injecting doses of up to 1.4×10^{13} particles into skeletal muscles of several mouse strains (C57BL/6, BALB/c, and SCID). *In vivo*, the CMV vector mediated higher levels of expression, with sustained serum levels over 800 $\mu\text{g/ml}$ in SCID and over 400 $\mu\text{g/ml}$ in C57BL/6 mice. These serum concentrations are 100,000-fold higher than those previously observed with AAV vectors in muscle and are at levels which would be therapeutic if achieved in humans. High level expression was delayed for several weeks but was sustained for over 15 wk. Immune responses were dependent upon the mouse strain and the vector dosage. These data suggest that recombinant AAV vector transduction of skeletal muscle could provide a means for replacing AAT or other essential serum proteins but that immune responses may be elicited under certain conditions.

Alpha-1-antitrypsin (AAT) deficiency is the second most common monogenic lung disease, accounting for 3% of all early deaths due to obstructive pulmonary disease. AAT is produced in the liver, secreted into the serum, and circulated to the lung where it protects elastin fibers and other connective tissue components of the alveolar wall from degradation by neutrophil elastase. Current therapy for AAT deficiency includes avoidance of cigarette smoke exposure and weekly *i.v.* infusions of recombinant human AAT (hAAT) protein (1). Attempts at gene augmentation have been limited by the short duration of expression and by the high circulating levels of AAT, which are required for therapeutic effect (800 $\mu\text{g/ml}$) (2).

Several groups have demonstrated that adeno-associated virus (AAV) vectors are capable of stable *in vivo* expression (3–5) and are less immunogenic than other viral vectors (6). AAV is a nonpathogenic human parvovirus whose life cycle includes a mechanism for long-term latency. In the case of wild-type AAV (wtAAV), this persistence is due to site-specific integration into a site on human chromosome 19 (AAVS1) (7), whereas with recombinant AAV (rAAV) vec-

tors, persistence occurs by both episomal persistence and integration into non-chromosome 19 locations (8–9). rAAV latency also differs from that of wtAAV in that wtAAV is rapidly converted to double-stranded DNA in the absence of helper virus (e.g., Ad) infection, whereas rAAV-leading strand synthesis is delayed in the absence of helper virus (10–11).

rAAV vector expression in skeletal muscle appears to be particularly robust and long-lived. Kessler *et al.* (5) demonstrated that murine skeletal myofibers transduced by an rAAV vector were capable of sustained secretion of biologically active human erythropoietin (hEpo), apparently without eliciting an immune response against hEpo. Likewise, expression and secretion of sustained therapeutic levels of leptin was observed in *ob/ob* mice after AAV muscle transduction (12). Even in this case, however, the level of expression observed was only in the range of 2–5 ng/ml. In the case of AAT, therapeutic serum levels of at least 800 $\mu\text{g/ml}$ will be required.

We sought to maximize rAAV vector expression to approach levels of AAT secretion require for therapy by increasing the dose and comparing a number of constitutively active promoters, including the cytomegalovirus (CMV) immediate early promoter, the human elongation factor 1- α promoter (EF1), and the small nuclear RNA promoters, U1a and U1b. *In vivo* experiments presented here demonstrate that stable therapeutic-range hAAT expression was achieved by using rAAV-skeletal muscle transduction but that immune responses were elicited under certain circumstances.

MATERIALS AND METHODS

Construction of rAAV Plasmids. The rAAV-AAT vector plasmids used for these experiments are shown (Fig. 1). In brief, the plasmid pN2FAT (13) plasmid was digested with *XhoI* to release 1.8-kb fragment containing the hAAT cDNA along with the simian virus 40 (SV40) promoter and polyA signal. This fragment was subcloned into pBlueScript (Stratagene) and, after the removal of the SV40 promoter by *HindIII* digestion and religation, the hAAT cDNA and polyA were released by *XbaI* and *XhoI* digestion. This 1.4-kb *XbaI*–*XhoI* fragment was then cloned into pTR-UF5 [AAV-inverted terminal repeat (ITR)-containing vector] between the *XbaI* site 3' to the CMV promoter and the *XhoI* site 5' to the polyoma enhancer/the HSV thymidine kinase promoter cassette, which drives *neo*. This yielded the pAAV-CMV-AAT construct (C-AT). Analogous constructs containing the U1a, U1b, and EF1 promoter were constructed similarly.

Packaging of rAAV Vectors. Vectors were packaged by using a modification of the method described by Ferrari *et al.* (14). Plasmids containing the AAV *rep* and *cap* genes (15) and the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/9514384-5\$2.00/0
PNAS is available online at www.pnas.org.

Abbreviations: AAV, adeno-associated virus; rAAV, recombinant AAV; AAT, alpha-1-antitrypsin; hAAT, human AAT; CMV, cytomegalovirus; SV40, simian virus 40; wt, wild-type; EF1, elongation factor 1- α promoter; Ad, adenovirus; i.u., infectious units.

§To whom reprint requests should be addressed. e-mail: flotttr@peds.ufl.edu.

FIG. 1. Transient transfection of C2C12 myoblasts with AAV-AAT. (Upper) Four AAV-AAT vector cassettes. The A-AT and B-AT constructs contain the promoters from the small nuclear RNA genes, U1a and U1b, respectively. The C-AT construct contains the CMV promoter, whereas the E-AT vector uses the human elongation factor 1- α (ELF in the figure) promoter. ITR, AAV inverted terminal repeat; An, polyA signal; Tk, the HSV thymidine kinase promoter; *neo*, the Tn5 neomycin phosphotransferase gene. (Lower) The rates of secretion from transfected cultures. C-AT does not differ significantly from E-AT, but both differ from A-AT and B-AT ($P < 0.05$).

Ad genes (E2a, E4, and VA-RNA) were cotransfected along with AAV-AAT vector plasmid into 293 cells grown in Cell Factories (Nalge Nunc). Cells were harvested and disrupted by freeze-thaw lysis to release virions that were purified by iodixanol gradient ultracentrifugation followed by heparin Sepharose affinity column purification (15). Vector preparations had physical titers assessed by quantitative competitive PCR and biological titers assessed by infectious center assay. The presence of wtAAV was likewise assayed with appropriate internal AAV probes. The high-dose C-AT stock had a particle-titer of 2.0×10^{14} particles/ml and an infectious titer of 5.0×10^{11} infectious units (i.u.)/ml (particle to i.u. ratio = 400:1). The low-dose C-AT measured 8×10^{12} particles/ml and 1.2×10^{10} i.u./ml (particle to i.u. = 667:1). For the E-AT experiments, the titers were 1×10^{13} particles/ml and 2.5×10^{10} i.u./ml (particle to i.u. = 400:1). The low-dose C-AT stock had a wt-like AAV particle titer (i.e., positive AAV genome PCR) equal to 0.1 times the recombinant titer but no detectable infectious wtAAV. The other two preparations had wt-like AAV particle titers $<10^{-5}$ times the recombinant titer and no detectable infectious wtAAV.

In Vitro Transfection and Transduction. C2C12 murine myoblasts were grown in 35-mm wells (4×10^5 cells/well) and transfected with 5 μ g of each plasmid DNA by using Superfect (Qiagen). Secretion of hAAT into the medium was assessed 2 days after transfection by using an antigen-capture ELISA

assay with standards which have been previously reported (2). An SV40 promoter *luciferase*-expression plasmid, pGL2 (Promega), was used as an internal control. For transduction experiments, cells were transduced with multiplicities of infection ranging from 4×10^5 to 4×10^6 particles per cell. Cultures were selected in geneticin (350 μ g/ml), and resistant clones were isolated for hAAT secretion studies.

In Vivo Injection of AAV-C-AT and AAV-E-AT Vectors into Murine Muscle. Mice strains (C57BL/6, SCID, and BALB/c) were obtained from Jackson Laboratories (Bar Harbor, ME) handled as approved by the University of Florida Animal Care Committee. Animals were anesthetized by metophane inhalation, and aliquots of vector were injected percutaneously into the quadriceps femoris muscles of both hind limbs. The volume of vector ranged from 50 to 100 μ l and the total amount of virus injected per animal ranged from 5×10^{10} to 1.4×10^{13} DNase-resistant particles.

Antigen Capture ELISA Assay for hAAT Expression. Microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 100 μ l of caprine anti-hAAT (1:200, Cappel/ICN) in Vollers buffer (overnight at 4°C). Standards and unknowns containing hAAT were incubated in the plate at 37°C for 1 hr. After blocking, a second antibody (1:1,000 rabbit anti-hAAT, Boehringer Mannheim) was reacted with the captured antigen. Detection was performed by using a third antibody incubation (1:800 caprine anti-rabbit IgG-peroxidase conjugate) followed by *o*-phenylenediamine (Sigma) detection by A_{490} .

ELISA Assay for Anti-hAAT and Anti-AAV VP3 Antibodies. Wells were coated with antigen (1 μ g of hAAT or 100 ng of VP3) at 4°C overnight, blocked, and then reacted with dilutions of either test serum or positive control antibodies at 37°C for 1 hr. After washing, a caprine-anti-mouse IgG-peroxidase conjugate was used as a secondary antibody (1:1,500 dilution) to detect bound anti-AAT antibody, using a standard *o*-phenylenediamine reaction, as above. Antibody levels were quantitated by comparison with monoclonal antibodies vs. VP3 and hAAT.

Lymphocyte Proliferation Assays to Detect Cell-Mediated Immune Responses. Lymphocyte proliferation assays were performed to detect T cell responses to the hAAT and VP3 antigens. Fresh splenocytes were grown in 96-well plates coated with 0, 0.1, 1, and 10 μ g of either hAAT or VP3 in RPMI-C+ medium. On day 3, a pulse of [3 H]thymidine was added, and the cells were harvested on day 4 for lysis and scintillation counting. Phytohemagglutinin (PHA) was used as a mitogen for positive control wells. A stimulation index was calculated for each antigen dosage level by dividing the cpm of [3 H]thymidine incorporated in the antigen-stimulated cells by the cpm in an unstimulated well.

RESULTS

In Vitro Studies in C2C12 Myoblasts. To determine the relative strength of a number of constitutively active promoters in the context of AAV-AAT vectors, we constructed AAV-AAT expression vectors containing the CMV, EF1, U1a, and U1b promoters (Fig. 1). Each of these was transfected into the murine C2C12 myoblast cell line. Both the EF1 and the CMV promoter were active for AAT expression, with EF1 construct (AAV-E-AT) expressing 850 ng/10⁵ cells/day and the CMV construct (AAV-C-AT) expressing $\approx$ 670 ng/10⁵ cells/day, as measured by a human-specific AAT ELISA assay (Fig. 1). The levels of expression from the U1a and U1b constructs were undetectable.

To characterize the level and duration of expression in the setting of vector transduction, C2C12 cells were transduced with either AAV-E-AT or AAV-C-AT at multiplicities of infection ranging from 4×10^5 to 4×10^6 particles per cell. Cells were then selected for expression of the *neo* gene

(present in each of the AAV constructs) by growth in G418-containing medium. Several cell clones and pooled cell populations were independently analyzed for AAT expression 4 wk later (Fig. 2). There was a trend toward higher levels of expression at higher multiplicities of infection, and the E-AT construct expressed at least 10-fold greater quantities under all conditions in these long-term cultures. The most active E-AT clone expressed hAAT at a rate of over 1,400 ng/10⁵ cells/day.

In Vivo Expression of hAAT from Murine Skeletal Muscle. To determine whether the AAV-AAT constructs would be active *in vivo* in muscle, doses of vector were injected into the quadriceps femoris muscle of mice. Circulating serum levels of hAAT were then measured for 11–15 wk along with four saline-injected controls from each strain. In the case of the C-AT vector (Fig. 3A), levels of expression were sufficient to achieve serum levels in excess of 800 μg/ml in SCID mice after a single injection of 1.4 × 10¹³ particles. A dose-effect relationship was observed, with expression levels in SCID being at least 20-fold lower at the 5 × 10¹¹ particle dose. The levels of expression increased over the first several weeks and were stable thereafter until the time of killing. Because hAAT has a half-life of <1 wk, this indicated continuous expression. Levels from C57BL/6 mice were comparable and also achieved values near the therapeutic range. Because these levels are higher than any previously reported with AAV vectors in muscle, we sought to confirm this by immunoblotting serum samples with anti-hAAT antibody (Fig. 3C). The intensity of reaction of the anti-hAAT antibody with the high-dose mouse sera was consistently higher than that seen with a 100 μg/ml hAAT control sample thus confirming that high level expression was achieved.

In similar studies, two of three BALB/c mice injected with 1 × 10¹¹ particles of the C-AT vector did not express hAAT at detectable levels (Fig. 4). Both of these were found to have high levels of anti-hAAT antibodies. Surprisingly, expression levels from the AAV-E-AT vector after *in vivo* injection were lower than those seen with the C-AT vector (Fig. 3B), with maximal levels of ≈250 ng/ml at the 5 × 10¹¹ dose at and beyond 7 wk in SCID mice. When the dose was further increased to 1 × 10¹² particles, levels of ≈1,200 ng/ml were observed and were once again stable for 15 wk after injection.

FIG. 2. Stable transduction of C2C12 myoblasts with AAV-AAT vectors *in vitro*. The mean rates of secretion from G418-resistant cultures 1 mo after transduction with either packaged E-AT vector or packaged C-AT vector are shown. In each instance, a “low” multiplicity transduction (4 × 10⁵ particles/cell) and a high multiplicity transduction (4 × 10⁶ particles/cell) were performed. E-AT “low” and “high” are greater than “high” multiplicity C-AT (*P* = 0.02) but are not significantly different from each other (*n* = 3).

FIG. 3. Sustained secretion of therapeutic levels of hAAT from the C-AT vector in either SCID or C57BL mice. (A) The mean total serum levels of hAAT observed in groups of either SCID (squares) or C57BL (circles) mice receiving either low dose (5 × 10¹¹ particles) (open symbols) or high dose (1.4 × 10¹³ particles) (filled symbols) single injections of the C-AT vector measured at time points ranging from 1 to 16 wk after injection. For each strain, the high-dose curve is significantly different from the low-dose curve (*P* = 0.009 for SCID, *P* = 0.02 for C57BL), but the strains do not differ from each other. (B) Analogous data with the E-AT vector. None of these differences were significant. (C) An immunoblot of sera taken from several of the C-AT vector-treated mice at 11 wk after vector administration. Ten microliters of a 1:100 dilution of serum was electrophoresed by 10% SDS/PAGE, blotted, and incubated with 1:1,500 dilution of goat anti-hAAT-horseradish peroxidase conjugate (Cappel/ICN). Samples from three high-dose SCID (h1–h3), one high-dose C57BL (h3), and three low-dose C57BL (lo1–lo3) were included, along with one negative control (saline-injected = sal) serum to indicate the level of reactivity with endogenous mAAT. As a standard, hAAT was added either to negative-control C57BL serum (first hAAT lane) or to PBS (second hAAT) lane to a final equivalent serum concentration of 100 μg/ml.

Levels were similar between SCID and immune competent C57BL/6 mice.

Immunologic Studies. In studies in BALB/c mice, antibody levels against hAAT were substantial in two of three animals injected (Fig. 4). The one which did not have circulating

FIG. 4. Some BALB/c mice mount humoral immune responses to hAAT, which correlate with lower serum levels but no observable

Genomic DNA				Episomal DNA				Control	
1	2	3	-	1	2	3	-	+	-

FIG. 5. Persistence of rAAV-AAT vector DNA in high molecular weight form. PCR products were amplified from DNA prepared by Hirt extraction from three SCID mice injected 16 wk earlier with 5×10^{11} resistant-particles of C-AT and analyzed by Southern blot. The high molecular weight Hirt pellet (genomic DNA lanes) and the low molecular weight supernatant (episomal DNA lanes) were analyzed separately. Control lanes include a sample in which an hAAT cDNA plasmid was the template DNA (+) and a control in which water was the template (-). In this internal PCR reaction, a 500-bp product is expected regardless of whether or not the vector genome is integrated.

anti-hAAT was the only animal with levels of hAAT expression similar to those in the C57BL/6 and SCID groups at the same dosage level. C57BL/6 and SCID mice receiving the same dose of vector did not produce any detectable humoral immune responses. The high-dose C57-C-AT injection group had detectable levels of antibody directed against VP3 and hAAT. In this case, the circulating anti-hAAT antibodies did not interfere with expression of circulating hAAT. To determine whether any cell-mediated immune responses were mounted, lymphocyte proliferation assays were performed by using either hAAT or AAV-VP3 for antigenic stimulation of primary splenic lymphocytes harvested at the time of animal sacrifice, 16 wk after vector injection. Using this method, no cellular immune responses were detectable in any of the mice.

Lack of Toxicity from Direct Vector Injection. To determine whether there was any direct toxicity, inflammation, or neoplastic change associated with vector injection, animals underwent complete necropsies, and histopathologic examination was performed on 5- μ m sections taken from the site of vector injection and from a panel of other organs, including the brain, heart, lungs, trachea, pancreas, spleen, liver, kidney, and jejunum. No histologic abnormalities were observed in any of these sites, even among those mice which developed humoral immune responses against hAAT (Fig. 4b).

Molecular Evidence of AAV-AAT Vector Persistence. To confirm the presence of vector DNA, a vector-specific PCR (*neo* primers 5'-TATGGGATCGGCCATTGAAC-3', and 5'-CCTGATGCTCTTC-GTCCAGA-3') was performed on DNA extracted from 3 SCID mice 16 wk after injection with the C-AT vector, and PCR products were analyzed by Southern blot analysis with a 32 P-labeled vector-specific probe (Fig. 5). We analyzed the state of vector DNA by using the Hirt

toxicity. (a) Serum hAAT levels (upper graph) and serum anti-hAAT antibody levels (lower graph) as determined by ELISA performed on serum taken from mice injected with 1×10^{11} particles of the C-AT vector. Each set of symbols represents an individual animal (□, no. 1; △, no. 2; ○, no. 3). Note the inverse correlation between the presence of antibody and the presence of circulating hAAT. (b) hematoxylin/eosin-stained sections from the injection site in BALB/c mice (original magnification, X2000). Section A is from the hAAT-expressing animal (animal no. 1 from a); section B is from an anti-hAAT-positive mouse which did not have appreciable hAAT activity in the serum (animal no. 2 from a). Section C is from the site of injection in a BALB/c mouse injected with a similar dose of the E-AT vector.

procedure to separate the low molecular weight episomal DNA from the high molecular weight fraction, which would contain integrated forms and large concatemers. In each case, vector DNA was present in the high molecular weight DNA fraction, whereas in only one of the animals was there a signal in the episomal fraction. This result indicates that by 16 wk most of the vector DNA in our animals was either integrated or in large concatemers.

DISCUSSION

Several gene therapy approaches for α 1AT-replacement have been evaluated *in vivo* including recombinant adenovirus (Ad), cationic liposomes, retroviral vectors, naked DNA injection, and gold-particle bombardment (16–20). Two studies demonstrated that myofibers are capable of secretion of biologically active hAAT into the serum (19, 20). Another study demonstrated that with a systemically delivered Ad-hAAT vector (predominantly to liver), the CMV promoter achieved the highest transient level of expression, 20 mg/ml, whereas the human EF1 promoter had the highest stable levels of expression, 2 mg/ml (21). Both of these are well above the threshold value for prevention of emphysema, which, as stated above, is 800 μ g/ml or 11 μ M. Although the first generation Ad vectors were limited by immunologic rejection of transduced cells, newer high capacity Ad vectors have proven to be less immunogenic and have achieved stable serum concentrations of $\approx$ 100 μ g/ml (22).

Our data, like earlier studies of rAAV in muscle, indicate that this system has several advantages. In recent studies, this system has proven to be efficient, stable, nontoxic, and relatively nonimmunogenic. Our results also are consistent with previous studies of rAAV vectors in muscle (4, 5), in that transcriptional down-regulation of CMV does not occur. Unexpectedly, *in vitro* experiments in the C2C12 myoblast line were not predictive of *in vivo* results. It is possible that transcription of EF1, which is required for active metabolism and proliferation, might be up-regulated in this cell line as compared with mature myofibers.

The kinetics of expression observed in our study are notable. The pronounced delay in the onset of expression suggests, as did earlier work (4, 5), that vector DNA is gradually converted into double-stranded forms by leading strand synthesis. After 7–9 wk, a plateau of expression was finally reached and expression was stable thereafter. This result may correspond with the time frame during which stable, high molecular weight forms of the vector genome were produced. The pattern of immune responses were interesting, in that they were strain-specific in the case of reactions to the hAAT transgene, and were dose-related in the C57BL/6 strain. BALB/c mice reacted strongly against hAAT even at low doses, and this limited the expression of circulating hAAT. Although no antibody responses were seen in BALB/c mice before 4 wk after dosing, this is not surprising given the lack of detectable circulating hAAT protein before this time point in other mice at the same dosage level.

Morral *et al.* (23), recently demonstrated a very similar strain-specific pattern of responses in the context of E2a-deleted Ad vectors, in that C57BL/6 mice did not react to hAAT whereas C3H mice did. The reason for the relatively nonimmunogenic nature of rAAV is not entirely known. In the current study, hAAT may be highly homologous to any of the five murine alpha-1-antitrypsin gene products (24), allowing for cross tolerance. It also is possible that the relative inability of rAAV vectors to transduce dendritic cells, as has been recently shown (6), could account for some of the advantage of rAAV over Ad in this case.

Overall, the current study indicates that with increasing doses of rAAV in muscle, serum levels of a therapeutic protein can be increased into the range of several hundred micrograms

per milliliter. Although this approach will need to be tested in larger animals, this data validates the concept of an AAV-based gene therapy for the prevention of emphysema due to AAT deficiency. Furthermore, these findings extend the range of serum protein concentrations achievable by gene augmentation, thus potentially extending the range of disorders treatable by such a therapeutic approach. The fact that a dose-response limit has not yet been observed indicates that further increases of expression may yet be achievable. Because humans are $\approx$ 1,000-fold larger by weight than mice, it is likely that significant scale-up will be required for clinical therapy of hAAT deficiency. As further advances in rAAV-packaging technology are developed, this hypothesis can be tested in future studies.

Funded by grants from the National Institutes of Health [HL59412, DK51809, and RR00082 (to T.F.) and HL59412 (to B.B.)]. Some of the authors may be entitled to royalties from products described herein.

- Wewers, M. D., Casolaro, M. A., Sellers, S. E., Swayze, S. C., McPhaul, K. M., Wittes, J. T. & Crystal, R. G. (1987) *N. Engl. J. Med.* **316**, 1055–1062.
- Brantly, M. L., Wittes, J. T., Vogelmeier, C. F., Hubbard, R. C., Fells, G. A. & Crystal, R. G. (1991) *Chest* **100**, 703–708.
- Flotte, T. R., Afione, S. A., Conrad, C., McGrath, S. A., Solow, R., Oka, H., Zeitlin, P. L., Guggino, W. B. & Carter, B. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10613–10617.
- Xiao, X., Li, J. & Samulski, R. J. (1996) *J. Virol.* **70**, 8098–8108.
- Kessler, P. D., Podsakoff, G., Chen, X., McQuiston, S. A., Colosi, P. C., Matelis, L. A., Kurtzman, G. & Byrne, B. J. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14082–14087.
- Jooss, K., Yang, Y., Fisher, K. J. & Wilson, J. M. (1998) *J. Virol.* **72**, 4212–4223.
- Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M. & Berns, K. I. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2211–2215.
- Afione, S. A., Conrad, C. K., Kearns, W. G., Chunduru, S., Adams, R., Reynolds, T. C., Guggino, W. B., Cutting, G. R., Carter, B. J. & Flotte, T. R. (1996) *J. Virol.* **70**, 3235–3241.
- Kearns, W. G., Afione, S. A., Fulmer, S. B., Caruso, J., Flotte, T. R. & Cutting, G. R. (1996) *Gene Ther.* **3**, 748–755.
- Fisher, K. J., Gao, G. P., Weitzman, M. D., DeMatteo, R., Burda, J. F. & Wilson, J. M. (1996) *J. Virol.* **70**, 520–532.
- Ferrari, F. K., Samulski, T., Shenk, T. & Samulski, R. J. (1996) *J. Virol.* **70**, 3227–3234.
- Murphy, J. E., Zhou, S., Giese, K., Williams, L. T., Escobedo, J. A. & Dwarki, V. J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 13921–13926.
- Garver, R. I., Jr., Chytil, A., Courtney, M. & Crystal, R. G. (1987) *Science* **237**, 762–764.
- Ferrari, F. K., Xiao, X., McCarty, D. M. & Samulski, R. J. (1997) *Nat. Med.* **3**, 1295–1297.
- Zolotukhin, S., Byrne, B. J., Mason, E., Zolotukhin, I., Summerford, C., Samulski, R. J. & Muzyczka, N. (1998) *Gene Therapy* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 12.
- Canonico, A. E., Conary, J. T., Meyrick, B. O. & Brigham, K. L. (1994) *Am. J. Respir. Cell Mol. Biol.* **10**, 24–29.
- Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Paakko, P. K., Gilardi, P., Stratford-Perricaudet, L. D., Perricaudet, M., *et al.* (1991) *Science* **252**, 431–434.
- Kay, M. A., Li, Q., Liu, T. J., Leland, F., Toman, C., Finegold, M. & Woo, S. L. (1992) *Hum. Gene Ther.* **3**, 641–647.
- Levy, M. Y., Barron, L. G., Meyer, K. B. & Szoka, F. C. (1996) *Gene Ther.* **3**, 201–211.
- Qui, P., Ziegelhoffer, P., Sun, J. & Yang, N. S. (1996) *Gene Ther.* **3**, 262–268.
- Guo, Z. S., Wang, L. H., Eisensmit, R. C. & Woo, S. L. (1996) *Gene Ther.* **3**, 802–810.
- Schiedner, G., Morral, N., Parks, R. J., Wu, Y., Koopmans, S. C., Langston, C., Graham, F. L., Beaudet, A. L., Kochanek, S. (1998) *Nat. Gen.* **18**, 180–183.
- Morral, N., O'Neal, W., Zhou, H., Langston, C. & Beaudet, A. (1997) *Hum. Gene Ther.* **8**, 1275–1286.
- Borriello, F. & Krauter, K. S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9417–9421.