JOURNAL OF VIROLOGY, Oct. 1999, p. 8027–8034 Vol. 73, No. 10
0022-538X/99/$04.00+0

Effects of Stuffer DNA on Transgene Expression from Helper-Dependent Adenovirus Vectors

ROBIN J. PARKS,[1,2]† JONATHAN L. BRAMSON,[2] YONGHONG WAN,[2] CHRISTINA L. ADDISON,[1]‡ AND FRANK L. GRAHAM[1,2]*

Departments of Biology[1] and Pathology,[2] McMaster University, Hamilton, Ontario L8S 4K1, Canada

Received 16 February 1999/Accepted 2 July 1999

We have analyzed transgene (*lacZ*) expression from a first-generation adenovirus (Ad) vector in comparison to helper-dependent (hd) Ads deleted for various portions of the viral coding sequences and generated by using the Cre/*lox*P helper-dependent system (R. J. Parks et al., Proc. Natl. Acad. Sci. USA 93:13565–13570, 1996). An hd vector deleted for approximately 70% of the Ad genome (AdRP1001) provided levels and durations of transgene expression similar to those of a control first generation Ad vector containing an identical expression cassette. Deletion of all Ad sequences from the hdAd and replacement with a ~22-kb fragment of lambda DNA resulted in a decrease in the level and duration of *lacZ* expression which could not be reversed by the inclusion of a matrix attachment region. However, substitution of the lambda stuffer in the fully deleted hdAd with sequences from the human hypoxanthine-guanine phosphoribosyltransferase gene resulted in significantly improved transgene expression. In vitro assays for cytotoxic T lymphocytes (CTL) directed against putative peptides encoded by the vector backbone showed that, although CTL were generated against the vector containing the lambda DNA, no such CTL were generated against the vector containing the hypoxanthine-guanine phosphoribosyltransferase (HPRT) sequences. Surprisingly, the rate of loss of the HPRT- and lambda-containing vectors from mouse liver was similar, despite the differences in expression kinetics, indicating that the lambda stuffer-directed CTL were inefficient at eliminating the transduced cells. Thus, the nature of the DNA backbone of hdAds can have important effects on the functioning of the vector. Since most fully deleted vectors require "stuffer" DNA as part of the vector backbone to maintain optimum vector size, these observations must be taken into account in the design of hdAd vectors.

Adenoviruses (Ads) have received considerable attention for use in gene therapy because of their relatively large cloning capacity, their ease of genetic manipulation and growth, and their ability to transduce many different tissue types containing both replicating and nonreplicating cells (6, 29). However, first-generation Ad vectors, which are rendered replication defective by deletion of E1, have proven to be inadequate for the long-term, stable expression of transgene which is necessary for the correction of most genetic diseases. Many researchers have identified factors which may contribute to this poor performance, including the use of foreign versus self transgenes (42, 45, 56, 62), strong innate and inflammatory responses to the vector (60, 61), acute and chronic toxicity due to low level viral gene expression from the vector backbone (45, 46), and the generation of anti-Ad cytotoxic T lymphocytes (CTL) as a consequence of either de novo viral gene expression (11, 63, 64, 66) or perhaps processing of peptides contained in the virion (31). It is likely that decay of transgene expression is due to a combination of several, or all, of these factors.

In an attempt to increase the efficacy of Ad-mediated transgene delivery, several methods have been described to blunt the anti-Ad immune response. Transient inhibition of immune responses or induction of tolerance can overcome these hurdles, extending transgene expression and allowing repeated administration of first-generation vectors (30, 32, 34, 38, 40, 51, 57, 60, 62, 67). However, complications and potential side effects may make immune suppression impractical for widespread clinical use. Second-generation Ad vectors, which contain further deletions or mutations in other regions of the genome, such as E2 or E4, have been shown to further attenuate the expression of viral proteins (3, 13–15, 17, 20, 21, 65); however, it is unclear whether these vectors have an increased effectiveness in vivo (13, 15, 41).

Recently, we and others have developed helper-dependent systems for the generation of Ad vectors deleted for most or all adenovirus protein coding sequences (16, 27, 33, 37, 39, 44, 48). Such helper-dependent Ad (hdAd) vectors need retain only those *cis*-acting elements required for virus replication and packaging, as all proteins are provided in *trans* by a helper virus. Early attempts to develop hdAd systems were relatively unsuccessful due to the high levels of contaminating helper virus, low recovery, and poor stability of vector during vector propagation (16, 26, 33, 37, 44). Nevertheless, preliminary studies of in vivo gene transfer and expression mediated by hdAd provided very encouraging results (7, 9).

To prevent the packaging of helper virus, we developed the Cre/*lox*P helper-dependent system which involves the use of a helper virus containing a packaging signal flanked by *lox*P sites (48). Upon infection of a 293-derived cell line that stably expresses the bacteriophage P1 Cre recombinase (8), the packaging signal is excised from almost all the helper virus DNA, rendering its genome unpackageable. The helper virus retains the ability to replicate and provides all of the functions required in *trans* for the replication and packaging of an hdAd. This system facilitates the generation of high-titer hdAd preparations with substantially reduced quantities of contaminating

* Corresponding author. Mailing address: Department of Biology, McMaster University, 1280 Main St., West, LSB-430, Hamilton, Ontario L85 4K1, Canada. Phone: (905) 525-9140, ext. 23545. Fax: (905) 521-2955. E-mail: graham@mcmaster.ca.

† Present address: Centre for Molecular Medicine, Ottawa Hospital Research Institute, Ottawa, Ontario K1H 8L6, Canada.

‡ Present address: Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109-0360.

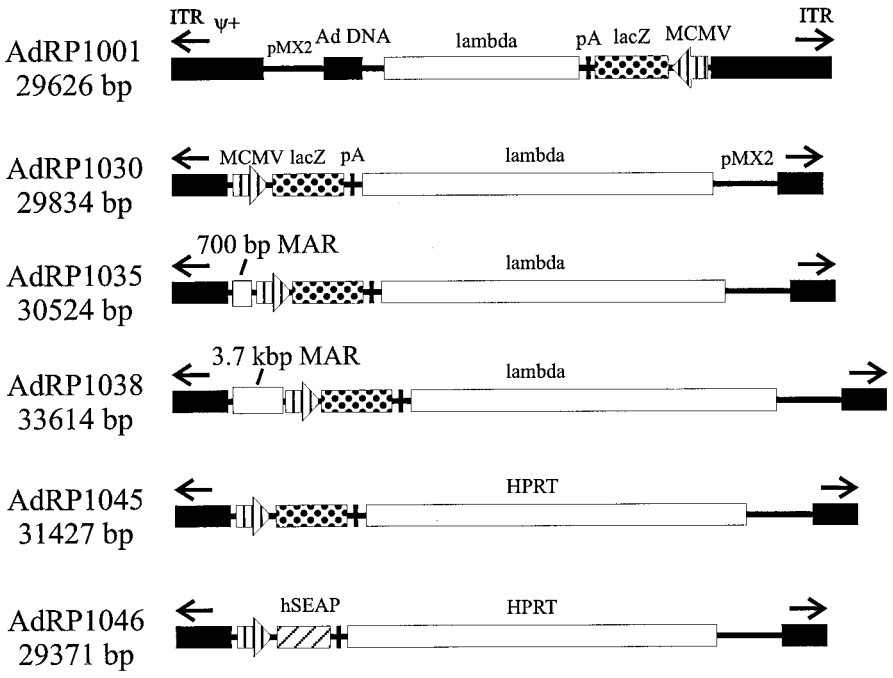

FIG. 1. Schematic structure of helper-dependent Ad vectors. AdRP1001 is a 29.6-kb helper-dependent Ad vector deleted for Ad coding sequences between 5,789 and 29,792 bp and encodes the *E. coli* β-Gal gene (*lacZ*) under the regulation of the MCMV immediate-early promoter/enhancer (MCMV) and simian virus 40 polyadenylation sequence (pA). The E1 region of AdRP1001 is disrupted by the insertion of a bacterial origin of replication and ampicillin resistance gene (pMX2 [22]) at 1,338 bp of the conventional Ad5 sequence. AdRP1001 also contains an 8.4-kb bacteriophage lambda DNA (31,617 to 39,888 bp of the conventional lambda map). AdRP1030 is deleted of all Ad protein coding sequences and contains the same *lacZ* expression cassette as AdRP1001 and a ~22-kb fragment of lambda DNA (415 to 22,425 bp of lambda). AdRP1035 is similar in structure to AdRP1030, but with the addition of a 692-bp DNA fragment containing the MAR from intron 1 of the HPRT gene inserted at a unique *Xba*I site located immediately upstream of the MCMV promoter of pRP1030. AdRP1038 is also similar to AdRP1030, but contains a 3.7-kb *Hin*dIII fragment from the HPRT first intron (containing the MAR) inserted at the unique *Xba*I site of pRP1030. AdRP1045 is similar in structure to AdRP1030 but contains a 22-kb fragment from the human HPRT locus replacing the lambda DNA. AdRP1046 is similar in structure to AdRP1045 but is deleted of a 1.2-kb *Stu*I fragment from the HPRT sequence and encodes a cDNA for human secreted alkaline phosphatase gene in place of the *lacZ* gene.

helper virus. HdAds can provide long-term, high-level transgene expression in vivo (47, 53), with significantly reduced vector-directed immune responses compared to first-generation vectors (46, 47). An important feature of the helper-dependent system is that all virion components, except the virion DNA, derive from the helper virus and thus genetically identical vectors can be generated simply by switching the serotype of the helper virus. Thus, should transgene expression decrease over time, the use of hdAds of alternative serotypes will permit effective readministration of a vector with the identical genotype (49).

In the present study, we have analyzed expression from vectors deleted of 70 to 100% of the Ad coding sequences. Due to the requirement for maintaining the vector size within the limits for efficient DNA packaging, approximately 75 to 105% of the wild-type genome length (5, 50), these deletions must be replaced by "stuffer" segments of DNA. We show that the nature of the stuffer segment can have a significant influence on transgene expression.

MATERIALS AND METHODS

Cell and virus culture. All cell culture media and reagents were obtained from Gibco Laboratories (Grand Island, N.Y.). 293 cells (23) were grown in monolayer in F-11 minimum essential medium supplemented with 100 U of penicillin per ml, 100 mg of streptomycin per ml, 2.5 mg of fungizone per ml, and 10% fetal bovine serum for cell maintenance or 5% horse serum for virus infection. Recombinant Ad helper viruses were grown and titers were determined on 293 cells, as previously described (28). The 293-derived cell line that stably expresses the Cre recombinase, 293Cre4 (8), was propagated in complete F-11 medium supplemented with 0.4 mg of G418 per ml.

AdRP1001 is an hdAd deleted for sequences between 16 and 83 map units of the Ad genome and contains the *Escherichia coli* β-galactosidase (β-Gal) gene under the regulation of the murine cytomegalovirus immediate-early promoter and simian virus 40 polyadenylation sequence (Fig. 1). AdRP1001 also contains an 8.4-kb bacteriophage lambda DNA fragment to ensure that the vector is within the Ad5 DNA packaging constraints (50). AdRP1030 is deleted of all Ad protein coding sequences, encodes a *lacZ* expression cassette identical to that of AdRP1001, and contains an ~22-kb fragment of lambda DNA (415 to 22425 bp of the conventional lambda map). AdRP1035 is similar in structure to AdRP1030 but with the addition of a 692-bp DNA fragment containing the matrix attachment region (MAR) from intron 1 of the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (55). The HPRT MAR was amplified from pSTK117, a plasmid containing a 16-kb fragment of HPRT genomic DNA including the first intron (kindly provided by Stefan Kochanek, University of Cologne, Cologne, Germany), by PCR with synthetic oligonucleotides 5′-GAG CCTAGGCCCATGTCCATCGAATGAG and 5′-GAGTCTAGATGAGGTC AGGA-GATGGAG and was inserted at a unique *Xba*I site located immediately upstream of the murine cytomegalovirus (MCMV) promoter. AdRP1038 is also similar to AdRP1030 but contains a 3.7-kb *Hin*dIII fragment from the HPRT first intron (containing the MAR) inserted at the unique *Xba*I site of pRP1030. AdRP1045 is similar in structure to AdRP1030 but contains a 22-kb fragment from the human HPRT locus replacing the lambda DNA. AdRP1046 is similar in structure to AdRP1045 but is deleted of a 1.2-kb *Stu*I fragment from the HPRT sequence and encodes a cDNA for human secreted alkaline phosphatase gene (hSEAP; Tropix) in place of the *lacZ* gene. The HPRT genomic sequence was obtained from Andrew J. Bett (Merck Research Laboratories, West Point, Pa.). All hdAd vectors were amplified by using the AdLC8cluc helper virus (E1/E3-deleted virus) in 293Cre4 cells, as previously described (48, 50), and the DNA structures of all hdAd vectors were confirmed by restriction digestion analysis of DNA isolated from virions. AdCA35 is a first-generation Ad vector in which the E1 region is replaced by a *lacZ* expression cassette identical to that of AdRP1001 (1). The titer of each vector was determined on 293 cells as the number of transducing particles, or "blue-forming units" (BFU), per ml. Total particle counts were determined spectrophotometrically ($1A_{260} = 1.1 \times 10^{12}$ particles/ml). For AdRP1030, the stock used in these experiments contained 6.6×10^{11} particles/ml, 5.2×10^{9} BFU/ml, and 1.1×10^{6} PFU/ml, resulting in a particle/BFU ratio of 120:1 and a helper virus contamination of approximately 0.02%, as calculated by PFU/BFU × 100%. For AdRP1045, the stock used in

these experiments contained 5.5×10^{11} particles/ml, 5.6×10^{10} BFU/ml, and 7.5×10^{6} PFU/ml, resulting in a particle to BFU ratio of 10:1 and a helper virus contamination of approximately 0.01%.

In vitro expression studies. Semiconfluent 60-mm dishes of A549 cells (10^6 cells per dish) were transduced with 10^6 BFU of vector for 30 min at 37°C and then overlaid with 5 ml of medium. At the indicated times postinfection, the cells were washed twice with phophate-buffered saline (PBS), overlaid with Reporter Lysis Buffer (Promega), and the resulting cell lysate and cellular debris collected in a microfuge tube. The cell lysates were stored at −70°C until the end of the experiment, at which time the lysates were thawed and vortexed briefly, and the cellular debris were pelleted by microcentrifugation. For both in vitro and in vivo expression studies, duplicate time points were assayed, and the average of the two values is reported. Protein samples from time course experiments were prepared and assayed at the end of the experiment. Repeat experiments showed similar results, and only one representative data set is presented. The assays for β-Gal activity were performed by using a standard colorimetric assay involving *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate essentially as described by Miller (43) or a chemiluminescent assay (Boehringer Mannheim). The quantity of β-Gal present in each sample was calculated by comparison with a standard curve generated from serial dilution of purified β-Gal protein or is simply reported as relative light units (RLU).

Animals and in vivo expression studies. Six- to eight-week-old FVB/n female mice were obtained from Harlan Laboratories. The *lacZ*-transgenic mice used in this study were an outbred strain (FVB/n × BALB/c) which encoded a hemizygous copy of the *lacZ* gene under the regulation of the myoD promoter, as originally described by Goldhamer et al. (18, 19). Mice were injected through the tail vein with 10^8 BFU of vector and, at the indicated times postinjection, the mice were euthanized, the livers were removed, and the tissue was frozen at −70°C until the end of the experiment.

Crude protein lysates from liver were prepared for β-Gal assays as follows. The liver was placed in 3 ml of PBS, homogenized by using a Tekmar Tissumizer (one-third maximum intensity, approximately 15 s), and sonicated by using a Branson Sonifier (one-third maximum intensity, two times with 15-s bursts). The resulting crude lysates were cleared of cellular debris by a 5-min centrifugation at 2,000 rpm in a Beckman GPR centrifuge. The lysates were then heated for 15 min at 50°C. This heating step causes the majority of the proteins in the sample, but not the bacterial *lacZ*, to denature, and the denatured proteins were removed by microcentrifugation at 16,000 × *g* for 10 min, resulting in lysates cleared of all particulate material. The cleared lysates were assayed for β-Gal as described above.

CTL assays. CTL assays were performed on splenocytes isolated from treated or control mice essentially as described previously (59). Spleens were removed from immunized mice at the indicated times after vector injection, and the splenocytes were dissociated and stimulated with gamma-irradiated PTO516 cells infected at a multiplicity of infection (MOI) of 20 of AdCA35 (β-Gal specific) or AdRP1030 ("lambda" specific) or 2,000 particles per cell of AdRP1046 ("HPRT" specific) for 5 days at a ratio of 100:1. Target cells for the ^{51}Cr-release assay were prepared by infecting PTO516 cells for 24 h with an MOI of 50 of AdCA35, AdRP1030, or AdRP1046 (5,000 particles/cell) or were mock infected with PBS; cells were then labeled with $Na^{51}CrO_4$ (^{51}Cr, 100 mCi/2 × 10^6 cells; New England Nuclear). The effector cells (i.e., cocultured splenocytes) were harvested, counted, and mixed in V-bottomed microtiter dishes with target cells at various effector/target ratios. After a 6-h incubation at 37°C, ^{51}Cr release was measured by a gamma counter, and the specific release was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%.

Detection of vector DNA. The quantity of vector DNA in the livers of treated mice was determined by using semiquantitative PCR. Briefly, total DNA was isolated from homogenized liver samples by using DNAzol (Life Technologies) as recommended by the manufacturer. Aliquots of the liver DNA (1 μg) was subjected to 30 rounds of PCR amplification with the synthetic oligonucleotides 5′-ACCCTGGCGTTACCCAACTTA and 5′-CTGCACCATCGTCTGCACATC, which amplify a 1,049-bp product from the *lacZ* gene of all hdAds used in this study. Equal aliquots of the PCR reaction were separated on a 0.8% agarose gel, and the resulting DNA bands were quantitated by using ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, Md.). To confirm that equal quantities of total liver DNA were included in each reaction, control PCR reactions were performed with synthetic oligonucleotides which amplified a β-actin product.

RESULTS

In vitro expression from AdRP1001 versus AdCA35. As a result of the poor duration of transgene expression generally associated with first-generation Ads, several groups of investigators have hypothesized that further attenuation of these vectors, either by deletion or mutation of other essential regions, might improve the expression characteristics of Ad vectors. We therefore constructed an hdAd, designated AdRP1001, that was deleted of approximately 70% of the Ad protein coding sequences but that contained an 8-kb fragment of lambda DNA as stuffer and a *lacZ* expression cassette. We analyzed transgene expression from AdRP1001 compared to a first-generation Ad vector with an identical transgene expression cassette, AdCA35. Although AdRP1001 induced up to sixfold-higher levels of expression in A549 or primary rat hepatocytes in vitro (data not shown), the levels and kinetics of expression from AdRP1001 and AdCA35 were virtually identical in vivo. Mice were injected intravenously with 10^8 BFU of AdRP1001 or AdCA35, and the livers were removed from euthanized animals at various times postinjection. Crude protein extracts prepared from the tissues were assayed for β-Gal expression by using a chemiluminescent assay as described in Materials and Methods. For AdRP1001, peak levels of β-Gal were observed at 24 h posttransduction ($7.05 \pm 2.10 \times 10^9$ RLU/tissue, $n = 4$) and declined to background levels by 12 days ($1.08 \pm 0.22 \times 10^6$ RLU/tissue). The peak of expression for AdCA35 ($6.06 \pm 2.52 \times 10^9$ RLU per tissue, $n = 4$) was quantitatively similar to that observed for AdRP1001 and again, after it reached a maximum at 3 days post-transduction, expression declined to background levels by day 12. Similar results were observed in tumor tissue in a mouse model of mammary adenocarcinoma (data not shown). These data indicate that, in immunocompetent animals, AdRP1001 was initially capable of producing high levels of transgene expression but that, as observed with a first-generation Ad vector, transgene expression rapidly decreased to background levels by 2 weeks postinjection. As expected, we could detect anti-β-Gal CTL in FVB/n mice immunized with AdRP1001 or AdCA35; however, this loss of vector expression was apparently not due to immune responses to the transgene product, since no improvement in the duration of transgene expression was observed in *lacZ* transgenic or BALB/c *scid* mice (data not shown). These results suggest that, although immune responses to the transgene may have been involved, at least in part, in the elimination of expression from AdCA35- and AdRP1001-transduced FVB/n mice, other factors must also be responsible.

Transgene expression from a "fully deleted" hdAd. In contrast to the transient transgene expression observed in mice with AdRP1001, in other studies it was found that hdAd vectors deleted of all Ad protein coding sequences can provide long-term transgene expression in vivo of at least 40 weeks (53). Thus, it is possible that the Ad5 sequences retained in AdRP1001 were somehow preventing the long-term persistence of transgene expression. To determine whether deletion of all viral coding sequences was sufficient to improve transgene expression, we constructed a second hdAd, designated AdRP1030, deleted of all Ad protein coding sequences but containing the MCMV-*lacZ* expression cassette and a ~22-kb fragment of lambda DNA as stuffer. Unexpectedly, we found that cells transduced with this new vector expressed β-Gal at significantly reduced levels compared to AdRP1001 (Fig. 2A). The peak of expression from AdRP1030 occurred at day 1 post-transduction and expression declined thereafter, whereas, AdRP1001 produced increasing quantities of β-gal over the duration of the experiment, reaching levels several orders of magnitude higher than those observed for AdRP1030 (0.5 versus 1.4×10^3 ng of β-Gal per 10^6 cells). In vivo, AdRP1030 produced about 12-fold-less β-Gal within the liver of transduced animals than did AdRP1001 ($3.1 \times 10^8 \pm 1.65 \times 10^8$ versus $3.6 \times 10^9 \pm 0.56 \times 10^9$ RLU per tissue, respectively), and expression did not persist (Fig. 2B). Thus, deletion of all Ad protein coding sequences did not, in itself, confer persistent expression from an hdAd vector either in vitro or in vivo.

Effect of a matrix attachment region. Throughout the Ad lifecycle, the majority of the Ad DNA remains associated with

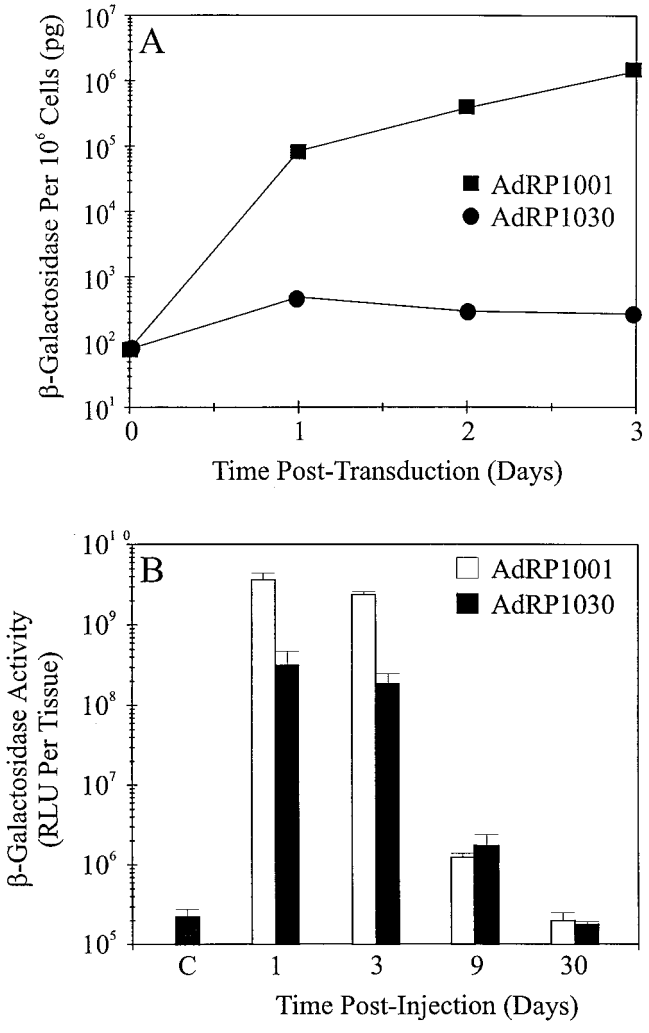

FIG. 2. In vitro and in vivo β-Gal expression from AdRP1001 and AdRP1030. (A) A595 cells were transduced with 10^6 BFU (MOI = 1) of AdRP1001 or AdRP1030. The cells were harvested at the times indicated and assayed for β-Gal. (B) Female FVB mice were injected intravenously with 10^8 BFU of AdRP1001 (open bars) or AdRP1030 (solid bars), and the quantity of β-Gal present in the liver was determined at various times after vector injection by using a chemiluminescence assay. Each datum point represents the average of two mice, and the bars represent maximum values.

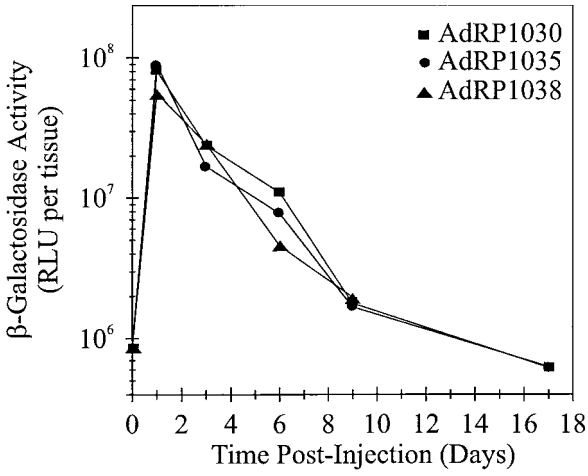

FIG. 3. Effect of a matrix attachment region on transgene expression from fully deleted hdAd. β-Gal activity in the liver of FVB/n mice was assayed at various times after intravenous injection of 10^8 BFU of AdRP1030, AdRP1035, or AdRP1038.

the nuclear matrix, suggesting a prominent role for this structure in virus transcription, replication, and assembly (4, 52). While it is clear that the interaction between the viral DNA and the nuclear matrix is mediated primarily by the adenovirus preterminal and terminal proteins (52) (note that, although the expression cassette for the pTp is removed from all hdAd used in this study, the terminal proteins are attached during viral replication in 293Cre cells and are provided in *trans* by the helper virus), it is possible that in deleting all Ad coding sequences we also removed other DNA segments which might be involved in mediating interactions with the nuclear matrix and conferring the high-level transgene expression typically noted for Ad vectors. Furthermore, inclusion of a matrix attachment region within retrovirus vectors can influence the persistence of transgene expression (2), and an hdAd containing an MAR has been shown to provide long-term, high-level expression of human α_1-antitrypsin (53). Therefore, we wished to determine if the inclusion of a MAR could "rescue" the poor expression associated with AdRP1030. Two additional hdAds were constructed, designated AdRP1035 and AdRP1038, that contained either a 0.7- or a 3.7-kb fragment, respectively, encompassing the MAR from HPRT (54). Inclusion of the MAR had no effect on the levels of β-Gal expression from AdRP1035 and AdRP1038 in A549 cells compared to AdRP1030 (data not shown). Additionally, the MAR did not have any significant effect on transgene expression in the liver of transduced FVB/n, where all three vectors induced expression of very similar quantities of β-Gal and with similar kinetics (Fig. 3). Thus, inclusion of a MAR in AdRP1030 did not enhance transgene expression.

Effect of stuffer. The lambda DNA used in AdRP1030 is essentially of prokaryotic origin. Since several studies have shown that certain cell types appear to be able to "detect" the nature of DNA (see Discussion), we next asked whether our choice of stuffer might be leading to poor expression from AdRP1030. We constructed a hdAd (AdRP1045) with the same *lacZ* expression cassette as AdRP1030 but containing a fragment of HPRT as stuffer instead of lambda. Initially, we transduced 60-mm dishes of A549 cells with 10^6 BFU of AdRP1030 or AdRP1045 and then examined the level of β-Gal expression at 24-h intervals. We observed that the peak levels of β-Gal expression within transduced A549 cells were significantly improved for AdRP1045 compared to AdRP1030 (85 versus 15 ng per 10^6 cells or sixfold higher; Fig. 4A). In vivo, although AdRP1045 and AdRP1030 induced similar maximal levels of transgene expression in the liver, AdRP1045 expressed longer and was still approximately 10-fold above background at the end of the assays (30 days postinjection; Fig. 4B). Similar results were observed in the *lacZ* transgenic animals used to avoid CTL against LacZ, although in this case the expression levels for AdRP1045 remained constant to the end of the experiment (day 16 postinjection; data not shown). These results suggest that the nature of the stuffer DNA within the hdAd can have a significant effect on transgene expression.

Stuffer-directed CTL. One explanation for the differences in expression kinetics of AdRP1030 versus AdRP1045 in vivo may be the production of peptides from the vector backbone which contribute to the induction of an immune response against the transduced cells leading to their eventual elimination. We therefore designed experiments to test for CTL di-

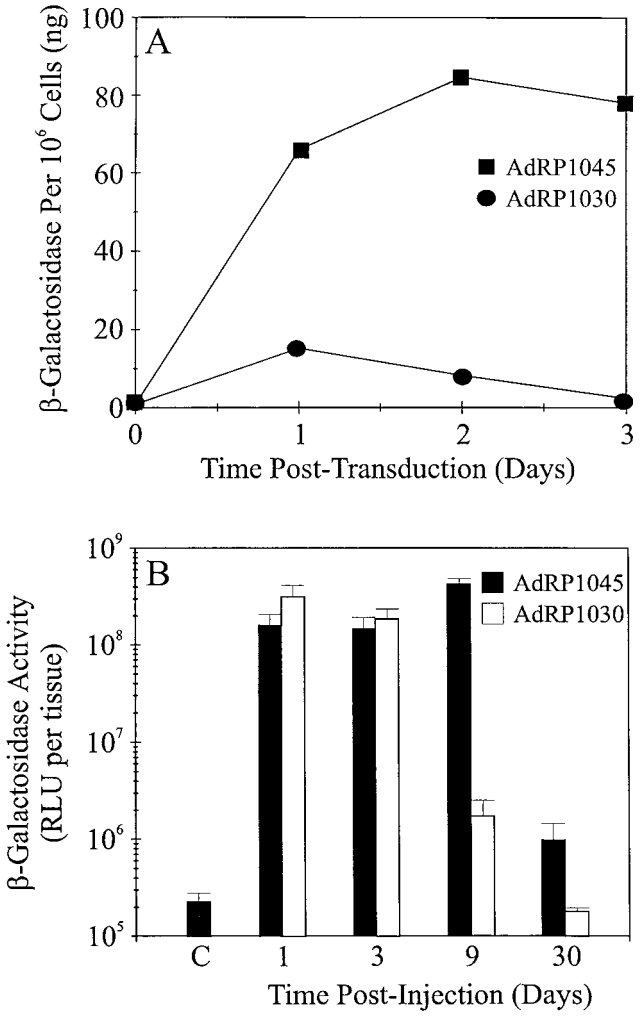

FIG. 4. Transgene expression from AdRP1030 and AdRP1045. (A) A549 cells were transduced with 10^6 BFU of AdRP1030 or AdRP1045 and assayed at various times posttransduction for β-Gal activity. (B) FVB/n mice were transduced intravenously with 10^8 BFU of AdRP1030 or AdRP1045, and the livers were removed from euthanized animals at various times postinjection and assayed for β-Gal activity.

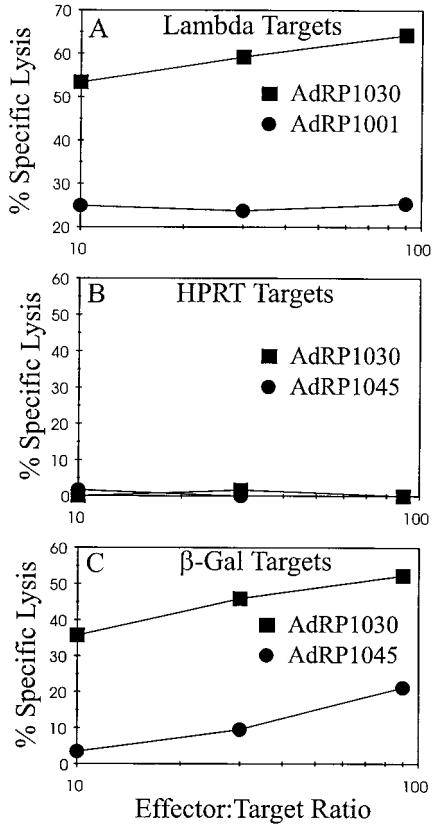

FIG. 5. Generation of CTL in mice immunized with AdRP1001. AdRP1030 or AdRP1045. *LacZ* transgenic (A) or FVB/n (B and C) were immunized with 10^8 BFU of AdRP1001, AdRP1030, or AdRP1045, and splenocytes from these animals were assayed for the presence of lambda-, β-Gal-, or HPRT-specific CTL as described in Materials and Methods. CTL were generated by coculturing splenocytes with PTO516 cells transduced with AdRP1030 (A), AdRP1046 (B), or AdCA35 (C). CTL were tested for cytotoxicity on ^{51}Cr-labeled target cells as follows: lambda (PTO516 transduced with AdRP1030, MOI = 50), β-Gal (PTO516 infected with AdCA35, MOI = 50), or HPRT (PTO516 transduced with AdRP1046, 5,000 particles per cell).

rected against peptides produced from the vector backbone of AdRP1030 or AdRP1045. In the first experiment, *lacZ* transgenic animals were injected with 10^8 transducing particles of AdRP1001 or AdRP1030. Splenocytes from these animals were then restimulated in coculture with PTO516 cells transduced with AdRP1030 and used in ^{51}Cr-release assays with AdRP1030-transduced targets. It is important to emphasize that AdRP1030 and AdRP1001 do not contain overlapping lambda DNA fragments. As shown in Fig. 5A, we observed lysis of target cells with restimulated splenocytes from AdRP1030-injected animals but not from animals treated with AdRP1001 or from naive animals (data not shown). Thus, it appeared that peptides were produced from the lambda backbone which were capable of eliciting a CTL response.

In a second experiment, FVB/n mice were injected with 10^8 BFU of AdRP1030 or AdRP1045, and splenocytes from these animals were restimulated in coculture with PTO516 cells transduced with AdCA35 (β-Gal stimulators) or AdRP1046, an hdAd vector identical to AdRP1045 but encoding a cDNA for hSEAP in place of the *lacZ* gene (HPRT stimulators). Since the FBV/n mice were not previously exposed to hSEAP and therefore would not have CTL precursors to the protein, we could use AdRP1046 as a source for stimulating putative backbone-specific CTL (i.e., HPRT specific). The restimulated splenocytes were then used in a ^{51}Cr-release assay with AdCA35- or AdRP1046-transduced PTO516 targets. We did not observe lysis of AdRP1046-transduced target cells from restimulated splenocytes from AdRP1030- or AdRP1045-injected animals (Fig. 5B), although similar assays for β-Gal-directed CTL were positive for both vectors (Fig. 5C), indicating that the animals had indeed been immunized with these vectors. It is unclear why the maximum level of β-Gal-directed killing for AdRP1045 was less than that observed for AdRP1030 (21% specific lysis versus 52%), since both vectors produce similar levels of β-Gal protein in transduced mice (Fig. 4B), but an intriguing possibility is that this difference may reflect adjuvant effects due to the apparent immunogenicity of the AdRP1030 backbone (see Discussion). Therefore, we conclude that, although vector-backbone-directed CTL appear to be generated against lambda-encoded peptides, the HPRT fragment used as a stuffer in AdRP1045 shows no comparable ability to induce CTL.

Persistence of hdAd DNA in the absence of transgene expression. To determine whether the loss of transgene expres-

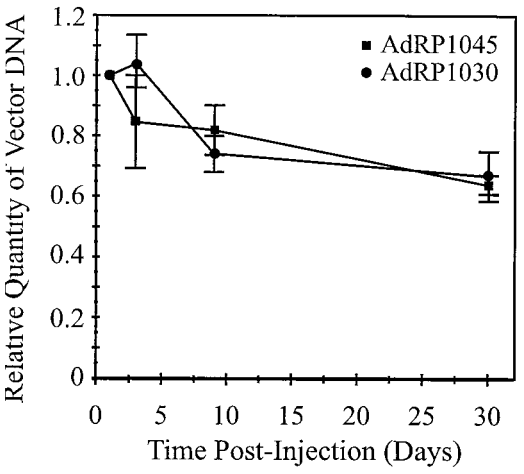

FIG. 6. PCR analysis of vector persistence in mouse liver. Aliquots (1 μg) of total mouse liver DNA was subjected to PCR amplification by using synthetic oligonucleotides specific to the *E. coli lacZ* gene. The resulting DNA products were separated on an agarose gel and quantitated. Densitometric values are presented as a fraction of the day 1 quantities. Each point represents the average of two mice, which were analyzed in independent experiments.

sion from AdRP1030 was due to elimination of the vector DNA from transduced hepatocytes due to the action of anti-lambda CTL, we isolated total liver DNA from mice which had been injected through the tail vein with 10^8 BFU of AdRP1030 or AdRP1045 and used semiquantitative PCR to determine the relative quantity of vector DNA within the liver at various times posttransduction. Consistent with the differences in the virion particle/transducing unit ratio for AdRP1030 (120:1) and AdRP1045 (10:1), we observed approximately a 10-fold difference in signal intensity between the two vectors at all time points (data not shown); however, the relative rate of loss of vector DNA was similar for the two vectors (Fig. 6). At the end of the 1-month time course, approximately 35% of the vector DNA was lost from the livers of animals treated with either vector compared to day 1 levels, a finding consistent with the observations of others (41, 61). Perhaps most strikingly, although similar quantities of DNA remained for both vectors at 9 days postinjection (approximately 80% of the day 1 levels), there was a 100-fold difference in the levels of transgene expression between AdRP1030 and AdRP1045 (Fig. 4B). Thus, although CTL were indeed generated to peptides produced of the lambda, but not HPRT, stuffer sequence, these CTL were ineffective at eliminating the AdRP1030-transduced cells, as has been observed by others (58). Moreover, our data suggest that there exists a fundamental difference(s) between the stuffer DNAs present in the two vectors which results in the elimination of expression from AdRP1030 or permits enhanced expression from AdRP1045.

DISCUSSION

We have shown that hdAd deleted for most or all Ad protein coding sequences can provide high levels of transgene expression, although the effectiveness of hdAd appears to be sensitive to the type of DNA used as stuffer. Use of lambda DNA resulted in reduced expression in vitro and in vivo compared to a vector containing HPRT sequences. Therefore, we conclude that, in order to optimize gene transfer and expression from hdAd vectors, the nature of the stuffer DNA, as well as the transgene sequences, must be carefully considered.

An hdAd deleted of approximately two-thirds of Ad protein coding sequences was capable of providing high levels of transgene expression both in vitro and in vivo. The level and kinetics of β-Gal expression in murine liver and tumor tissue were equivalent to those observed for a first-generation Ad vector, although transgene expression from both vectors was only transient. These results are in clear contrast with the results of Lieber et al. (39), who constructed a vector deleted for ~75% of Ad coding sequences and observed that transgene expression declined to undetectable levels much more rapidly than for a first-generation Ad vector. This instability may have been a consequence of the reduced DNA size of the vector generated by Lieber et al. (ca. 9 kb). We have determined that the Ad virion has a lower limit for efficient DNA packaging of approximately 27 kb (50), and it is possible that packaging of a 9-kb vector results in virion instability, for example, due to altered virion structure, and may somehow lead to a reduced efficiency of DNA transduction.

Vectors deleted of all viral coding sequences have recently been shown to provide long-term expression of both α_1-antitrypsin (53) and leptin (47). However, in the present study, we observed that deletion of all Ad protein coding sequences, in itself, was not sufficient to permit long-term expression. Although the vector described previously (53) contained an MAR, it appears that this element alone may not be sufficient for persistent expression since its inclusion in our lambda backbone vector did not alter transgene expression. The difference in expression between AdRP1030 and AdRP1045 was not due to "helper" functions provided by the small quantities of helper virus present in our hdAd preparation, since the hdAd doses of each vector (10^8 transducing units) contained approximately equal quantities of helper virus (~10^4 PFU per dose).

The nature of the stuffer DNA segment, which in many cases must be included in hdAd vectors in order to increase the size above the lower limit for efficient DNA packaging, may have a significant influence on transgene expression. Inclusion of a fragment of lambda DNA led to poor transgene expression in vivo, whereas an HPRT-derived stuffer provided significantly improved expression, suggesting that there are DNA-related "factors" which are involved in enhancing transgene expression from AdRP1045 or, alternatively, reducing expression from AdRP1030. For example, it is possible that the segment of HPRT that we used may contain an enhancer-like element which can increase expression from the MCMV promoter.

Another intriguing explanation for the poor performance of the lambda stuffer vector arises from the observation that some mammalian cell types are sensitive to the origin of foreign DNA. Introduction of bacterial DNA into macrophages, B lymphocytes, or NK cells results in cellular activation and the induction of inflammatory genes, which is not observed with eukaryotic DNA (10, 36, 54). This response is thought to be due, at least in part, to differences in CpG methylation and/or base composition. Indeed, depending on the context, a CpG motif can be either stimulatory (CpG-S) or inhibitory to immune activation (35). The lambda DNA contained within AdRP1030 encodes 91 CpG-S motifs, whereas only 7 CpG-S motifs are present in the HPRT fragment of AdRP1045. CpG-S motifs can also enhance CTL responses to proteins (12), and this may explain why we observed a stronger CTL response to β-Gal from mice treated with AdRP1030 compared to AdRP1045 (Fig. 5C). While DNA-mediated immune stimulation has only been reported for hematopoietic cells, it is possible that basic differences in the type and number of CpG motifs, DNA base composition, or secondary structure may be detected by many cell types, resulting in promoter downregulation, compartmentalization, and/or elimination of the DNA. Interestingly, the overall base composition of lambda (57%

G+C) is similar to Ad5 (55% G+C), which is substantially different from mammalian DNA (41% G+C [40% G+C for the HPRT fragment in AdRP1045]), suggesting that, if the cell is able to "detect" foreign DNA based on base composition, first- and second-generation Ad vectors and AdRP1030 may be susceptible to a similar fate (i.e., transient transgene expression) even in the absence of any viral gene expression. Alternatively, Chisari and coworkers have recently described a mechanism functioning in the liver whereby viral DNA is eliminated by a noncytopathic means which involves a change in the cytokine profile in the liver microenvironment (24, 25). It is possible that a similar mechanism is functioning in the liver of mice treated with AdRP1030, although with the difference that this phenomenon is sensitive to the nature of the DNA (i.e., lambda versus HPRT) and that, in our case, gene expression is reduced without vector DNA elimination.

Previous work with hdAd suggests that vectors deleted of all coding sequences have the potential to provide an efficient gene delivery vehicle with an increased cloning capacity and increased safety for gene therapy. However, the results presented here indicate that further study is clearly necessary for the optimal design and use of such vectors.

ACKNOWLEDGMENTS

We thank Carole Evelegh and John Rudy for excellent technical assistance and Michael A. Rudnicki for providing the *lacZ* transgenic mice. We also thank Stefan Kochanek and Andrew J. Bett for providing the HPRT genomic fragment and for valuable discussion and Dennis E. Bulman for technical advice.

This work was supported by grants from the National Institutes of Health, Natural Sciences and Engineering Research Council (NSERC), Medical Research Council (MRC), and the National Cancer Institute of Canada (NCIC) and by Merck Research Laboratories. R.J.P. was an NSERC and MRC Postdoctoral Fellow (PDF). J.L.B. was an MRC PDF, C.L.A. was a research student of the NCIC supported by funds provided by the Canadian Cancer Society, and F.L.G. was a Terry Fox Research Scientist of the NCIC.

REFERENCES

1. **Addison, C. L., M. Hitt, D. Kunsken, and F. L. Graham.** 1997. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. J. Gen. Virol. **78:**1653–1661.
2. **Agarwal, M., T. W. Austin, F. Morel, J. Chen, E. Bohnlein, and I. Plavec.** 1998. Scaffold attachment region-mediated enhancement of retroviral vector expression in primary T cells. J. Virol. **72:**3720–3728.
3. **Armentano, D., C. C. Sookdeo, K. M. Hehir, R. J. Gregory, St, G. A. Prince, S. C. Wadsworth, and A. E. Smith.** 1995. Characterization of an adenovirus gene transfer vector containing an E4 deletion. Hum. Gene Ther. **6:**1343–1353.
4. **Besse, S., and F. Puvion-Dutilleul.** 1994. Compartmentalization of cellular and viral DNAs in adenovirus type 5 infection as revealed by ultrastructural in situ hybridization. Chromosome Res. **2:**123–135.
5. **Bett, A. J., L. Prevec, and F. L. Graham.** 1993. Packaging capacity and stability of human adenovirus type 5 vectors. J. Virol. **67:**5911–5921.
6. **Bramson, J. L., F. L. Graham, and J. Gauldie.** 1995. The use of adenoviral vectors for gene therapy and gene transfer in vivo. Curr. Opin. Biotechnol. **6:**590–595.
7. **Chen, H. H., L. M. Mack, R. Kelly, M. Ontell, S. Kochanek, and P. R. Clemens.** 1997. Persistence in muscle of an adenoviral vector that lacks all viral genes. Proc. Natl. Acad. Sci. USA **94:**1645–1650.
8. **Chen, L., M. Anton, and F. L. Graham.** 1996. Production and characterization of human 293 cell lines expressing the site-specific recombinase Cre. Somat. Cell. Mol. Genet. **22:**477–488.
9. **Clemens, P. R., S. Kochanek, Y. Sunada, S. Chan, H. H. Chen, K. P. Campbell, and C. T. Caskey.** 1996. In vivo muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes. Gene Ther. **3:**965–972.
10. **Cowdery, J. S., J. H. Chace, A. K. Yi, and A. M. Krieg.** 1996. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. J. Immunol. **156:**4570–4575.
11. **Dai, Y., E. M. Schwarz, D. Gu, W. W. Zhang, N. Sarvetnick, and I. M. Verma.** 1995. Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. Proc. Natl. Acad. Sci. USA **92:**1401–1405.
12. **Davis, H. L., R. Weeranta, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Krieg.** 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. **160:**870–876.
13. **Dedieu, J. F., E. Vigne, C. Torrent, C. Jullien, I. Mahfouz, J. M. Caillaud, N. Aubailly, C. Orsini, J. M. Guillaume, P. Opolon, P. Delaere, M. Perricaudet, and P. Yeh.** 1997. Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. J. Virol. **71:**4626–4637.
14. **Engelhardt, J. F., X. Ye, B. Doranz, and J. M. Wilson.** 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc. Natl. Acad. Sci. USA **91:**6196–6200.
15. **Fang, B., H. Wang, G. Gordon, D. A. Bellinger, M. S. Read, K. M. Brinkhous, S. L. Woo, and R. C. Eisensmith.** 1996. Lack of persistence of E1-recombinant adenoviral vectors containing a temperature-sensitive E2A mutation in immunocompetent mice and hemophilia B dogs. Gene Ther. **3:**217–222.
16. **Fisher, K. J., H. Choi, J. Burda, S. J. Chen, and J. M. Wilson.** 1996. Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. Virology **217:**11–22.
17. **Gao, G. P., Y. Yang, and J. M. Wilson.** 1996. Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. J. Virol. **70:**8934–8943.
18. **Goldhamer, D. J., B. P. Brunk, A. Faerman, A. King, M. Shani, and C. P. Emerson, Jr.** 1995. Embryonic activation of the myoD gene is regulated by a highly conserved distal control element. Development **121:**637–649.
19. **Goldhamer, D. J., A. Faerman, M. Shani, and C. P. Emerson, Jr.** 1992. Regulatory elements that control the lineage-specific expression of myoD. Science **256:**538–542.
20. **Goldman, M. J., L. A. Litzky, J. F. Engelhardt, and J. M. Wilson.** 1995. Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2a-defective recombinant adenoviruses: a preclinical toxicology study. Hum. Gene Ther. **6:**839–851.
21. **Gorziglia, M. I., M. J. Kadan, S. Yei, J. Lim, G. M. Lee, R. Luthra, and B. C. Trapnell.** 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. J. Virol. **70:**4173–4178.
22. **Graham, F. L.** 1984. Covalently closed circles of human adenovirus DNA are infectious. EMBO J. **3:**2917–2922.
23. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristic of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74.
24. **Guidotti, L. G., P. Borrow, A. Brown, H. McClary, R. Koch, and F. V. Chisari.** 1999. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. J. Exp. Med. **189:**1555–1564.
25. **Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari.** 1999. Viral clearance without destruction of infected cells during acute HBV infection. Science **284:**825–829.
26. **Haecker, S. E., H. H. Stedman, R. J. Balice-Gordon, D. B. Smith, J. P. Greelish, M. A. Mitchell, A. Wells, H. L. Sweeney, and J. M. Wilson.** 1996. In vivo expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. Hum. Gene Ther. **7:**1907–1914.
27. **Hardy, S., M. Kitamura, T. Harris-Stansil, Y. Dai, and M. L. Phipps.** 1997. Construction of adenovirus vectors through Cre-lox recombination. J. Virol. **71:**1842–1849.
28. **Hitt, M., A. J. Bett, C. L. Addison, L. Prevec, and F. L. Graham.** 1995. Techniques for human adenovirus vector construction and characterization. Methods Mol. Genet. **7:**13–30.
29. **Hitt, M. M., C. L. Addison, and F. L. Graham.** 1997. Human adenovirus vectors for gene transfer into mammalian cells. Adv. Pharmacol. **40:**137–206.
30. **Jooss, K., Y. Yang, and J. M. Wilson.** 1996. Cyclophosphamide diminishes inflammation and prolongs transgene expression following delivery of adenoviral vectors to mouse liver and lung. Hum. Gene Ther. **7:**1555–1566.
31. **Kafri, T., D. Morgan, T. Krahl, N. Sarvetnick, L. Sherman, and I. Verma.** 1998. Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. Proc. Natl. Acad. Sci. USA **95:**11377–11382.
32. **Kass-Eisler, A., L. Leinwand, J. Gall, B. Bloom, and E. Falck-Pedersen.** 1996. Circumventing the immune response to adenovirus-mediated gene therapy. Gene Ther. **3:**154–162.
33. **Kochanek, S., P. R. Clemens, K. Mitani, H. H. Chen, S. Chan, and C. T. Caskey.** 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. Proc. Natl. Acad. Sci. USA **93:**5731–5736.
34. **Kolls, J. K., D. Lei, G. Odom, S. Nelson, W. R. Summer, M. A. Gerber, and J. E. Shellito.** 1996. Use of transient CD4 lymphocyte depletion to prolong transgene expression of E1-deleted adenoviral vectors. Hum. Gene Ther. **7:**489–497.
35. **Krieg, A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis.** 1998. Sequence motifs in adenoviral

DNA block immune activation by stimulatory CpG motifs. Proc. Natl. Acad. Sci. USA **95:**12631–12636.
36. **Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman.** 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature **374:**546–549.
37. **Kumar-Singh, R., and J. S. Chamberlain.** 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. Hum. Mol. Genet. **5:**913–921.
38. **Kuzmin, A. I., M. J. Finegold, and R. C. Eisensmith.** 1997. Macrophage depletion increases the safety, efficacy and persistence of adenovirus-mediated gene transfer in vivo. Gene Ther. **4:**309–316.
39. **Lieber, A., C. Y. He, I. Kirillova, and M. A. Kay.** 1996. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. J. Virol. **70:**8944–8960.
40. **Lochmuller, H., B. J. Petrof, G. Pari, N. Larochelle, V. Dodelet, Q. Wang, C. Allen, S. Prescott, B. Massie, J. Nalbantoglu, and G. Karpati.** 1996. Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. Gene Ther. **3:**706–716.
41. **Lusky, M., M. Christ, K. Rittner, A. Dieterle, D. Dreyer, B. Mourot, H. Schultz, F. Stoeckel, A. Pavirani, and M. Mehtali.** 1998. In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. J. Virol. **72:**2022–2032.
42. **Michou, A. I., L. Santoro, M. Christ, V. Julliard, A. Pavirani, and M. Mehtali.** 1997. Adenovirus-mediated gene transfer: influence of transgene, mouse strain and type of immune response on persistence of transgene expression. Gene Ther. **4:**473–482.
43. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
44. **Mitani, K., F. L. Graham, C. T. Caskey, and S. Kochanek.** 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. Proc. Natl. Acad. Sci. USA **92:**3854–3858.
45. **Morral, N., W. O'Neal, H. Zhou, C. Langston, and A. Beaudet.** 1997. Immune responses to reporter proteins and high viral dose limit duration of expression with adenoviral vectors: comparison of E2a wild type and E2a deleted vectors. Hum. Gene Ther. **8:**1275–1286.
46. **Morral, N., R. J. Parks, H. Zhou, C. Langston, G. Schiedner, J. Quinones, F. L. Graham, S. Kochanek, and A. L. Beaudet.** 1998. High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. Hum. Gene Ther. **9:**2709–2716.
47. **Morsy, M. A., M. Gu, S. Motzel, J. Zhao, J. Lin, Q. Su, H. Allen, L. Franlin, R. J. Parks, F. L. Graham, S. Kochanek, A. J. Bett, and C. T. Caskey.** 1998. An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. Proc. Natl. Acad. Sci. USA **95:**7866–7871.
48. **Parks, R. J., L. Chen, M. Anton, U. Sankar, M. A. Rudnicki, and F. L. Graham.** 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc. Natl. Acad. Sci. USA **93:**13565–13570.
49. **Parks, R. J., C. M. Evelegh, and F. L. Graham.** Use of helper-dependent adenoviral vectors of alternative serotype permits repeat vector administration. Gene Ther., in press.
50. **Parks, R. J., and F. L. Graham.** 1997. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. J. Virol. **71:**3293–3298.
51. **Sawchuk, S. J., G. P. Boivin, L. E. Duwel, W. Ball, K. Bove, B. Trapnell, and R. Hirsch.** 1996. Anti-T cell receptor monoclonal antibody prolongs transgene expression following adenovirus-mediated in vivo gene transfer to mouse synovium. Hum. Gene Ther. **7:**499–506.
52. **Schaack, J., W. Y. Ho, P. Freimuth, and T. Shenk.** 1990. Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA. Genes Dev. **4:**1197–1208.
53. **Schiedner, G., N. Morral, R. J. Parks, Y. Wu, S. C. Koopmans, C. Langston, F. L. Graham, A. L. Beaudet, and S. Kochanek.** 1998. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nat. Genet. **18:**180–183.
54. **Stacey, K. J., M. J. Sweet, and D. A. Hume.** 1996. Macrophages ingest and are activated by bacterial DNA. J. Immunol. **157:**2116–2122.
55. **Sykes, R. C., D. Lin, S. J. Hwang, P. E. Framson, and A. C. Chinault.** 1988. Yeast ARS function and nuclear matrix association coincide in a short sequence from the human HPRT locus. Mol. Gen. Genet. **212:**301–309.
56. **Tripathy, S. K., H. B. Black, E. Goldwasser, and J. M. Leiden.** 1996. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. Nat. Med. **2:**545–550.
57. **Vilquin, J. T., B. Guerette, I. Kinoshita, B. Roy, M. Goulet, C. Gravel, R. Roy, and J. P. Tremblay.** 1995. FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. Hum. Gene Ther. **6:**1391–1401.
58. **Wadsworth, S. C., H. Zhou, A. E. Smith, and J. M. Kaplan.** 1997. Adenovirus vector-infected cells can escape adenovirus antigen-specific cytotoxic T-lymphocyte killing in vivo. J. Virol. **71:**5189–5196.
59. **Wan, Y., J. Bramson, R. Carter, F. Graham, and J. Gauldie.** 1997. Dendritic cells transduced with an adenoviral vector encoding a model tumor-associated antigen for tumor vaccination. Hum. Gene Ther. **8:**1355–1363.
60. **Wolff, G., S. Worgall, R. N. van, W. R. Song, B. G. Harvey, and R. G. Crystal.** 1997. Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. J. Virol. **71:**624–629.
61. **Worgall, S., G. Wolff, E. Falck-Pedersen, and R. G. Crystal.** 1997. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Hum. Gene Ther. **8:**37–44.
62. **Yang, Y., K. Greenough, and J. M. Wilson.** 1996. Transient immune blockade prevents formation of neutralizing antibody to recombinant adenovirus and allows repeated gene transfer to mouse liver. Gene Ther. **3:**412–420.
63. **Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.
64. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.
65. **Yang, Y., F. A. Nunes, K. Berencsi, E. Gonczol, J. F. Engelhardt, and J. M. Wilson.** 1994. Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. Nat. Genet. **7:**362–369.
66. **Yang, Y., Z. Xiang, H. C. Ertl, and J. M. Wilson.** 1995. Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. Proc. Natl. Acad. Sci. USA **92:**7257–7261.
67. **Zepeda, M., and J. M. Wilson.** 1996. Neonatal cotton rats do not exhibit destructive immune responses to adenoviral vectors. Gene Ther. **3:**973–979.