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Activation of Transgene Expression by Early Region 4 Is Responsible for a High Level of Persistent Transgene Expression from Adenovirus Vectors In Vivo

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The persistence of transgene expression has become a hallmark for adenovirus vector evaluation in vivo. Although not all therapeutic benefit in gene therapy is reliant on long-term transgene expression, it is assumed that the treatment of chronic diseases will require significant persistence of expression. To understand the mechanisms involved in transgene persistence, a number of adenovirus vectors were evaluated in vivo in different strains of mice. Interestingly, the rate of vector genome clearance was not altered by the complete deletion of early region 4 (E4) in our vectors. The GV11 ($E1^-E4^-$) vector genome cleared with a similar kinetic profile as the GV10 ($E1^-$) vector genome in immunocompetent and immunocompromised mice. These results suggest that the majority of adenovirus vector genomes are eliminated from transduced tissue via a mechanism(s) independent of T-cell, B-cell, and NK cell immune mechanisms. While the levels of persistence of transgene expression in liver or lung transduced with GV10 and GV11 vectors expressing β-galactosidase, cystic fibrosis transmembrane conductance regulator, or secretory alkaline phosphatase were similar in immunocompetent mice, a marked difference was observed in immunocompromised animals. Levels of transgene expression initially from both GV10 and GV11 vectors were the same. However, GV11 transgene expression correlated with loss of vector genome, while GV10 transgene expression persisted at a high level. Coadministration and readministration of GV10 vectors showed that E4 provided in *trans* could activate transgene expression from the GV11 vector genome. While transgene expression activity per genome from the GV10 vector is clearly activated, expression from a cytomegalovirus promoter expression cassette in a GV11 vector appeared to be further inactivated as a function of time. Understanding the molecular mechanisms underlying these expression effects will be important for developing persistent adenovirus vectors for chronic applications.

Adenovirus vectors provide an effective means to introduce new genes into cells. However, the initial robust expression of the newly introduced gene is transient (8). While persistent gene expression is not essential for all therapeutic uses of adenovirus vectors, it will be crucial for the treatment of chronic diseases. Therefore, a significant hallmark for vector evaluation is the chronicity of transgene expression in vivo.

Several different factors appear to effect transgene expression persistence in vivo. The current model for lack of persistence with adenovirus vectors is thought to be similar to the host response to wild-type virus infection. In wild-type infections, viral proteins are presented to the immune system, and over a period of several days to weeks, the cells expressing the viral antigens are recognized and eliminated. Several reported studies evaluating adenovirus vectors are consistent with this model and demonstrate persistent transgene expression in the absence of antigen-specific immunity (2, 9, 23, 25–27, 31, 32, 45, 51–60, 62). These results have suggested that the immunological response leading to the rapid loss of transgene expression most likely stems from expression of the transgene and production of viral antigens from the vector genome.

These immunological responses have been uncovered by using adenovirus vectors deleted in early region 1 (E1) and/or early region 3 (E3). To further reduce or completely eliminate the production of viral antigens, additional genome modifications have been constructed and tested. The extent to which these alterations and deletions affect immune system-related clearance mechanisms is not completely clear. The results from analyzing vectors with modifications that introduce only a small alteration in the E2A gene (13–15, 54, 55, 61) or from vectors that delete all or most of adenovirus genes (7, 30, 33) have been contradictory regarding transgene persistence in vivo. In addition, vector modifications that delete E1 and E4 have also shown contradictory results in regard to transgene persistence in vivo (1, 10, 16, 24). Taken together, these studies suggest that in vivo transgene persistence is a problem more complicated than initially suspected.

The deletion of E4 is especially important to the ultimate design of safe adenovirus vectors. Removal of the E4 region severely disrupts viral gene expression in transduced cells and eliminates several viral products that interact and antagonize cellular targets and processes. One of these E4 products, E4-ORF6, has been shown to block p53 function and to have oncogenic potential (11, 36). A similar cellular transforming or oncogenic function has also been linked to E4-ORF1 (19–22, 46–48). E4-ORF6 and E4-ORF3 have also been shown to be involved in altering mRNA expression at a posttranscriptional level (39–44). In addition, E4 products are involved in controlling the cellular transcription factor E2F (38), in E1A-induced p53-independent apoptosis (34), in modulating the phosphorylation status of cellular and viral proteins (28, 35) and have recently been shown to alter the nuclear transport of other proteins (17).

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The negative effects of expressing E4 region genes as well as viral late proteins have been eliminated in our E1$^-$E4$^-$ (GV11) series of vectors (4). Therefore, we compared genomes and transgene persistence profiles of the GV11 vector and of similar vectors containing wild-type E4 (GV10). The levels of persistence of GV11 vector genomes and transgene expression were similar to those for GV10 in immunocompetent animals. Similar vector genome persistence profiles were also seen in immunocompromised animals. However, the transgene expression profile for GV11 vectors was significantly altered in GV11 vector-transduced immunocompromised mouse lung and liver. These results suggest that adenovirus vector genomes are eliminated from transduced tissue via a mechanism independent of the well-described vector clearance models. Moreover, these analyses suggest that E4 products play a key role in the maintenance of transgene persistence in immunodeficient mice.

MATERIALS AND METHODS

Animal studies. All animal studies were carried out in accordance with the institutional guidelines of the Biomedical Research Institute and GenVec Inc., Rockville, Md. BALB/c, BALB/c nude, NCR nude, Swiss nude, and SCID/beige mice were purchased from Jackson Laboratories (Bar Harbor, Maine) or Taconic (Germantown, N.Y.), were mostly females, were approximately 6 to 8 weeks old at the time of initial vector administration, and were housed in a pathogen-free environment.

Adenovirus vectors and animal administration. Several different matched sets of adenovirus vectors were evaluated. Matched sets of vector stocks were used in this evaluation to ensure that the results obtained were due to deleting E4, not to differences in activity or the physical level of vector in each experiment. A matched set of vectors was defined as a GV10 (E1$^-$) vector and a GV11 (E1$^-$E4$^-$) vector that contained identical transgene expression cassettes, elicited similar dose-response curves in tissue culture for transgene expression, and had similar active-to-inactive particle ratios as determined by focus-forming unit assay and optical density reading at 260 nm, respectively.

The construction, production, and quantification of GV10 and GV11 vectors have been described previously (4). Vectors containing a Rous sarcoma virus (RSV) expression cassette in the E1 region that expressed β-galactosidase (AdRSVβgal.10 and AdRSVβgal.11) were evaluated. AdRSVβgal.11 contains a complete deletion of E4 as described elsewhere (4). Vectors containing a cytomegalovirus (CMV) expression cassette in the E1 region that expressed β-galactosidase (AdLacZ.10 and AdLacZ.11A) or that expressed secretory alkaline phosphatase (SEAP) (AdSEAP.10 and AdSEAP.11A) were also evaluated. AdLacZ.11A and AdSEAP.11A contain a genome structure similar to that of the previously described AdCFTR.11A (4). These vectors contain a complete deletion of E4 and the described E4 spacer for optimum production (4).

For all animal studies, vector stocks were purified through three sequential bandings on cesium chloride gradients. The purified capsids were dialyzed into 10 mM Tris (pH 7.8)–150 mM NaCl–10 mM $MgCl_2$–3% sucrose storage buffer, aliquoted, and stored at −80°C.

Prior to administration, vectors were prepared to the indicated dose by appropriate dilution with storage buffer. In all cases, vehicle represents this storage buffer with no adenovirus vector added. Vectors were administered intranasally for delivery to lung and intravenously (via tail vein) for delivery to liver. Animals were anesthetized with an intramuscular injection of anesthetic (Ketaset and Xylazine; Butler, Columbus, Ohio) prior to administration of a 75- to 100-μl total volume of vector.

Molecular analyses of tissue and serum. At the indicated time postadministration, a terminal dose of anesthetic was administered. The target tissue was then removed and washed in phosphate-buffered saline. The tissue was either flash frozen in liquid nitrogen and stored at −80°C for molecular analyses or prepared for histological analysis. Frozen tissue was pulverized with a mortar and pestle on dry ice, aliquoted into tubes, and stored at −80°C prior to extraction.

Total DNA was extracted by a modified proteinase technique (29). After restriction with *Kpn*I, viral DNA was detected by Southern blot analysis. A probe homologous to the adenovirus polymerase gene contained within the internal E2B region of adenovirus was used for optimized detection of vector DNA. Single-copy levels of detection were obtained by using the Southern protocols recommended for the Zeta-Probe GT membrane (Bio-Rad Laboratories, Hercules, Calif.). Total RNA was extracted with RNAzol or Ultraspec (Biotecx Laboratories, Houston, Tex.), and Northern analysis was performed (Zeta-Probe) with the indicated random-primed DNA probe (Stratagene, La Jolla, Calif.) specific for the RNA species noted.

Total protein was extracted in 1× reporter lysis buffer (Promega, Madison, Wis.) with the addition of 0.15 U of aprotinin per ml, 20 μM leupeptin, and 100 μg of phenylmethylsulfonyl fluoride per ml. Total protein content was determined with the Bio-Rad protein assay. β-Galactosidase activity was measured by chemiluminescence with Galacto-Light (Tropix, Bedford, Mass.). β-Galactosidase activity is expressed as relative light units per micrograms of protein. β-Galactosidase was detected histologically by washing the tissue extensively (six or more times) in phosphate-buffered saline, slicing the tissue to allow reagent access, staining with HistoMark 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate set (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), and fixing the tissue in Parafix (Molecular Histology Labs, Gaithersburg, Md.). The stained tissue was observed and photographed on a Nikon dissection scope.

For detection of SEAP in the serum, the animals were administered a light dose of anesthetic intramuscularly at the indicated time, and blood was collected via the ocular sinus. Serum was prepared, aliquoted, and stored at −80°C. SEAP expression levels were detected by chemiluminescence using Phospha-Light (Tropix). SEAP activity is expressed as nanograms of SEAP per milliliter of serum.

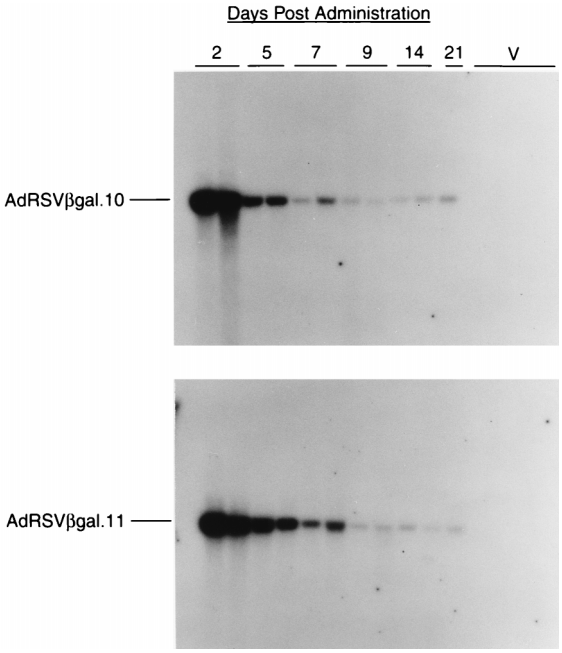

FIG. 1. Vector genome persistence in lung tissue of immunocompetent mice. BALB/c mice were administered 10^{11} total particles of AdRSVβgal.10 or AdRSVβgal.11 intranasally. At the indicated times postadministration, mice were sacrificed from each group. Total DNA was extracted from tissue and the level of viral DNA detected by Southern blot analysis. V, negative control animals that were administered only vehicle, no vector.

RESULTS

Vector persistence in immunocompetent mice. Viral protein expression is greatly reduced and viral replication potential is eliminated in cells transduced with the GV11 (E1$^-$E4$^-$) series of vectors compared to GV10 (E1$^-$) (3a, 4). Therefore, we thought that the GV11 vector would exhibit significantly longer transgene persistence in vivo, given its reduced immunogenic load.

The impact of complete E4 deletion in our GV11 adenovirus vectors was first tested in BALB/c mice following intranasal administration of matched GV10 and GV11 vector stocks. The first matched stocks of vectors expressed β-galactosidase from an RSV expression cassette contained within the E1 region of the vectors (AdRSVβgal.10 and AdRSVβgal.11). These analyses showed no difference between the two vector backbones in immunocompetent animals in vivo. Similar GV10 and GV11 vector genome clearance profiles were observed (Fig. 1). At 2 days postadministration, approximately 30 to 40% of the input GV10 or GV11 vector genomes were detected. Within 9 to 14 days postadministration, the level of vector genomes was re-

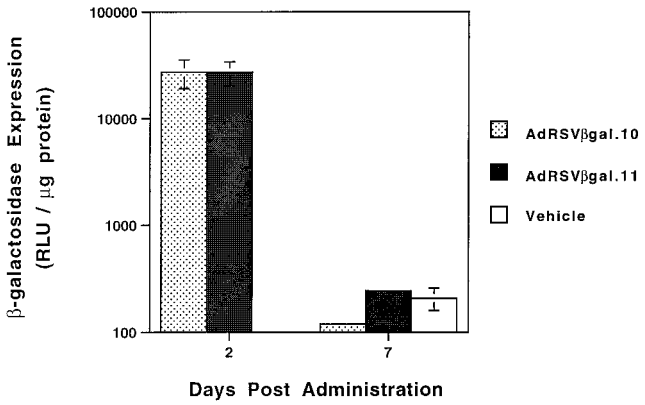

FIG. 2. Transgene expression in lung tissue of immunocompetent mice. BALB/c mice were administered 10^{11} total particles of AdRSVβgal.10 or AdRSVβgal.11 intranasally. At the indicated times postadministration, three mice were sacrificed from each group. Protein was extracted from tissue, and the level of β-galactosidase expression was detected by chemiluminescence. Error bars indicate standard error of the mean. RLU, relative light units.

duced to only ~1% of that administered. The kinetics of clearance and 30-fold reduction in vector DNA between 2 days and 2 weeks postadministration was also seen with vectors expressing β-galactosidase from a CMV expression cassette, human cystic fibrosis transmembrane conductance regulator from a CMV expression cassette and SEAP from a CMV expression cassette.

The level and persistence of transgene expression followed similar patterns for both GV10 and GV11 vectors in immunocompetent mice. As shown in Fig. 2, the levels of transduction were identical for the two vector genomes at 2 days postadministration. However, within 2 weeks postadministration, transgene expression was reduced at least 30-fold. Interestingly, a low level of vector DNA (~0.5 to 1% of input) was found to persist in the BALB/c lung throughout the time course of these experiments (4 to 6 weeks). A low level of transgene expression was also observed by quantitative reverse transcription-PCR (data not shown). The mechanism underlying low-level persistent vector genome and transgene expression is currently under investigation. In addition, similar GV10 and GV11 vector DNA and transgene expression persistence profiles were seen after intravascular administration to the liver and in a number of other immunocompetent strains of mice (data not shown). Taken together, these results suggest that clearance of vector genomes and transgene expression in lung or liver tissue of immunocompetent mice was not changed by deletion of the E4 region.

Vector persistence in immunodeficient mice. Transgene persistence can be influenced by several immunological factors in vivo. Some of these immune responses have been shown to result from expression of the transgene and production of viral antigens from the vector genome. To remove these variables, persistence profiles for GV10 and GV11 vectors were evaluated in immunodeficient mice. Figure 3 shows AdRSVβgal.10 and AdRSVβgal.11 vector genome persistence profiles in Swiss nude mice after intranasal administration to the lung. Similar genome clearance profiles were seen with these vectors as well as all other GV10 and GV11 vectors evaluated. At 2 days postadministration, 40 to 50% of the input particles could be accounted for, and within 14 days postadministration, a 5- to 10-fold reduction in vector DNA was detected. By 35 days postadministration, only 2 to 5% of input genomes remained.

These results show that vector genomes are cleared in the

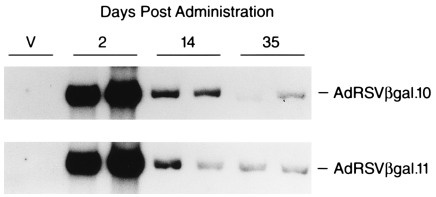

FIG. 3. Vector genome persistence in lung tissue of immunodeficient mice. NIH Swiss nude mice were administered 10^{11} total particles of AdRSVβgal.10 or AdRSVβgal.11 intranasally. At the indicated times postadministration, mice were sacrificed from each group. Total DNA was extracted from tissue, and the level of viral DNA was detected by Southern blot analysis. Lane V is as in Fig. 1.

immunodeficient mouse lung. Moreover, removal of the E4 region from the vector had no effect on vector DNA persistence. Compared to the results for immunocompetent mice, these results confirm that a T-cell-mediated response is involved in vector genome clearance. However, this type of immune mechanism appears to account for clearance of only a small part of the total particles administered to the lung. From these evaluations, it is apparent that larger portions of vector genomes are removed from the transduced tissue by alternative mechanisms. In addition, similar results comparing GV10 and GV11 vector genome persistence profiles were obtained after intravascular administration to the liver (data not shown). However, in these experiments the level of vector genome at 2 days postadministration was ~10-fold lower than that seen in the lung. These findings are consistent with those of Crystal and investigators (49, 50), who suggested that an innate clearance mechanism was responsible for substantial elimination of vector genomes and that this mechanism was more pronounced in the liver than in the lung.

While genome persistence profiles appeared to be similar for both GV10 and GV11 vectors, transgene expression was considerably different. The levels of β-galactosidase expression from AdRSVβgal.10 and AdRSVβgal.11 were similar at 2 days postinfection (Fig. 4). At 14 and 35 days postadministration, β-galactosidase expression remained at the 2-day level for AdRSVβgal.10. However, β-galactosidase in AdRSVβgal.11 transduced lung dropped to baseline levels at 14 days. Similar results were also obtained with $E1^-$ and $E1^-E4^-$ vectors (Ad-

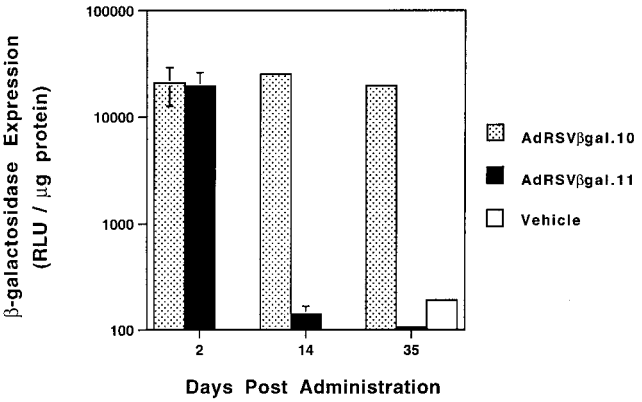

FIG. 4. Transgene expression in lung tissue of immunodeficient mice. NIH Swiss nude mice were administered 10^{11} total particles of AdRSVβgal.10 or AdRSVβgal.11 intranasally. At the indicated times postadministration, three mice were sacrificed from each group. Protein was extracted from tissue, and the level of β-galactosidase expression detected by chemiluminescence. Error bars indicate standard error of the mean. RLU, relative light units.

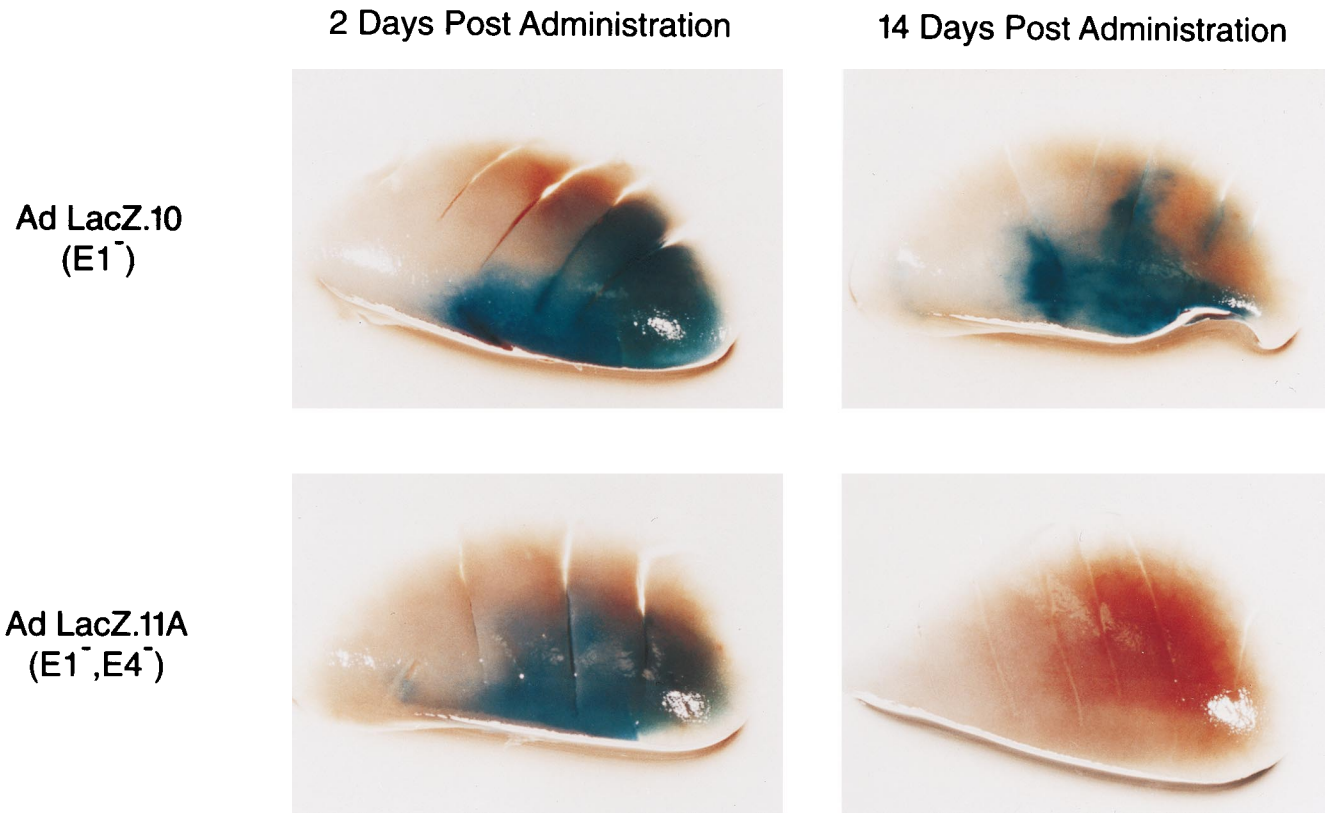

FIG. 5. Transgene expression in lung tissue of immunodeficient mice. NIH Swiss nude mice were administered 10^{11} total particles of AdLacZ.10 or AdLacZ.11A intranasally. At the indicated times postadministration, the mice were sacrificed and the presence of β-galactosidase expression was detected by staining.

LacZ.10 and AdLacZ.11A) expressing β-galactosidase from a CMV immediate-early promoter expression cassette (Fig. 5). It is incongruous that vector genomes are actively cleared but expression remains constant. Therefore, the GV10 vector genomes at 2 to 5 weeks postadministration somehow differ in the ability to express transgene compared to 2 days postadministration. One possible explanation is that the high-level transgene persistence seen with $E1^-$ adenovirus vectors in nude mice is due to E4-induced activation of transgene expression over time.

To explore this possibility and to test if the E4 activation was occurring at a transcriptional level, we compared RNA expression of a polymerase III (PolIII) gene product from the vector

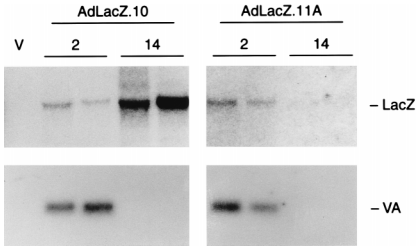

FIG. 6. RNA expression in lung tissue of immunodeficient mice. NIH Swiss mice were administered 10^{11} total particles of AdLacZ.10 or AdLacZ.11A intranasally. At the indicated times postadministration, two mice were sacrificed from each group. Total RNA was extracted from tissue, 20 μg of total RNA was loaded per lane, equal loading was confirmed by detection of ribosomal bands, and the level of message was detected by Northern analysis with DNA probes specific to either LacZ or VA RNA. Lane V is as in Fig. 1.

(VA RNA) to that of the PolII transgene expression cassette (LacZ). Total RNA was isolated from AdLacZ.10- and AdLacZ.11A-transduced lung tissue at 2 and 14 days postadministration, and Northern analysis was performed (Fig. 6). The levels of LacZ mRNA at 2 days postadministration were similar in AdLacZ.10- and AdLacZ.11A-transduced tissue, as suspected from the β-galactosidase activity determinations. At 14 days postadministration, LacZ mRNA levels not only were maintained in AdLacZ.10-transduced lung but were three- to fourfold higher than the 2-day level. As expected, the level of LacZ mRNA from AdLacZ.11A-transduced lung was below detection at 14 days postadministration. Interestingly, the levels of VA RNA were similar for GV10 and GV11 vectors and correlated with the levels of vector genome. Therefore, the transcriptional potential of the GV10 transgene expression cassette appears to be enhanced relative to the GV11 transgene expression cassette. These results imply that transcriptional activation of the transgene expression cassette appears to be critical for persistence from a GV10 vector.

Modulation of transgene expression in immunodeficient mice. To further explore the implications of these results, the level of transgene expression per genome was quantitated after intranasal administration of AdSEAP.10 and AdSEAP.11A in different immunodeficient strains of mice. AdSEAP.10 and AdSEAP.11A express a modified SEAP gene from a CMV promoter expression cassette (GenVec) (3). Vector DNA and SEAP levels were quantitated 2 and 21 days postadministration, and the results are represented as relative transgene expression per vector genome (Table 1). In this analysis, if the amount of transgene expression per vector genome is constant,

TABLE 1. Relative transgene expression per vector genome in immunodeficient mice

Vector	Time postadministration	Relative SEAP expression/vector DNA[a]		
		NIH Swiss nude	BALB/c nude	SCID/beige
AdSEAP.10	2 days	16	10	40
AdSEAP.10	3 wk	195	110	150
AdSEAP.11A	2 days	31	16	30
AdSEAP.11A	3 wk	0.1	2	0.6

[a] Vector DNA was detected from 10 μg of total DNA by Southern blot analysis and quantitated with a Packard Instant Imager as counts per minute per band. At the indicated times postadministration, serum was collected, and SEAP expression was detected by chemiluminescence and quantitated as nanograms of SEAP per milliliter of serum.

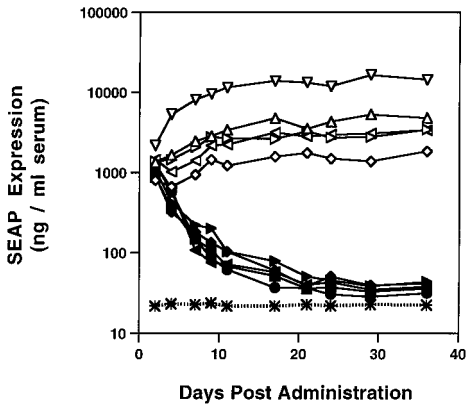

FIG. 7. Comparison of GV10 and GV11 transgene persistence after IV administration in the nude mouse. NIH Swiss nude mice were administered 10^{11} total particles of AdSEAP.10 or AdSEAP.11A intravenously. At the indicated times postadministration, serum was collected and SEAP expression was detected by chemiluminescence. Open symbols represent animals that received AdSEAP.10; closed symbols represent animals that received AdSEAP.11A; cross-hatched symbol and dashed line represent an animal that received only vehicle (no vector) and denotes the baseline for this assay.

the relative level of transgene expression per vector genome will remain the same over time.

In all mouse strains examined, the absolute amount of vector genome decreased over time for both GV10 and GV11. As expected, the level of transgene expression per GV10 genome increased ~10-fold over time in all strains of nude mice. The level of transgene expression also increased (~4-fold) in C.B-17/GbmsTac-scid-bgfDF N7 (SCID/beige) mice. SCID/beige mice carry the SCID mutation, which causes a lack of both T and B lymphocytes; and also carry the beige mutation that results in cytotoxic T-cell defects, macrophage defects, and NK cell function impairment. Therefore, a mechanism(s) other than that involving these immune pathways appears to be involved in the majority of vector genome clearance from transduced tissue.

Interestingly, the relative level of transgene expression per AdSEAP.11A vector genome did not remain constant. Relative transgene expression from the CMV-SEAP transgene was significantly lower (>8-fold) by 21 days postadministration. This result suggests that in addition to a significant loss of vector genome, the CMV expression cassette in a GV11 vector backbone is also inactivated.

E4 products transactivate gene expression. To further explore the role of E4 products in transgene expression persistence in vivo, we compared our vectors in the liver. For these experiments, we chose SEAP as the transgene marker because of (i) ease of transgene expression detection and (ii) the ability to monitor transgene expression persistence in individual animals. AdSEAP.10 or AdSEAP.11A was intravascularly administered via tail vein to five NIH Swiss nude animals, and the level of SEAP expression in the serum of each animal was monitored for 1 month (Fig. 7). As expected, SEAP expression from AdSEAP.10 administered animals remained high for the duration of the experiment. The level of SEAP expression from AdSEAP.11A decreased steadily from the level at 2 days postadministration, reaching ~5% of the level at 2 days postadministration after 3 to 4 weeks. The amount of viral DNA was equivalent for both vectors at all time points examined (data not shown). Therefore, the role that E4 plays in transgene persistence does not appear to be limited to the lung.

All of these results imply that E4 products activate transgene expression from adenovirus vector genomes. To test this model, NIH Swiss nude mice were coadministered AdSEAP.11A with AdLacZ.10. As seen in Fig. 8, the presence of E4 products in *trans* provided from AdLacZ.10 genomes maintained high SEAP expression levels from the AdSEAP.11A vector genome throughout the duration of the experiment (~5 months). Moreover, activation by E4 products was shown in readministration experiments where NIH Swiss nude mice were first administered AdSEAP.11A and then after 79 days readministered AdLacZ.10 (Fig. 8). Taken together, these experiments clearly show that E4 products act in *trans* to activate transgene expression from adenovirus vector genomes. It is interesting that the reactivation of the GV11 genome is not immediate. After readministration, it takes several days to reach the high level of expression found with GV10 alone. A part of this slow accumulation may be due to the long half-life of SEAP in serum (10 to 20 h [3a]). In addition, GV10 transgene expression appears to decrease over time. Whether this is due to normal cellular turnover and gradual loss of vector DNA or to a mechanism of inactivation similar to that seen with GV11 transgene expression has yet to be determined.

DISCUSSION

Studies with adenovirus vectors have shown that their severe limitation is in the persistence of transgene expression. This lack of persistence is probably due, at least in part, to activation of cellular immune responses by the expression of viral and transgene products leading to the ultimate death and clearance of the transduced cell. Vector modifications that reduce viral antigen presentation have been contradictory with regard to transgene persistence (1, 7, 10, 13–16, 24, 30, 33, 54, 55, 61). Our results suggest that an important variable in these studies may be the presence or absence of viral gene products (for example, from the E4 region) that modulate transgene persistence.

Our evaluation of $E1^-$ and $E1^-E4^-$ vectors has shown that only a small portion of the administered vector genome persists in vivo. Only 0.5 to 1% or 2 to 5% of the input particles persist in lung tissue of immunocompetent or immunodeficient mice, respectively. Therefore, even in an immunodeficient animal, at least 95% of the vector genomes are cleared. These results imply that the majority of vector genomes are cleared via a mechanism(s) independent of the well-described cytotoxic response. Within the detection limitations of these experiments, this genome clearance appears to be independent of initial vector dose (3a), which suggests that this mechanism may be a natural process probably tied to normal viral removal.

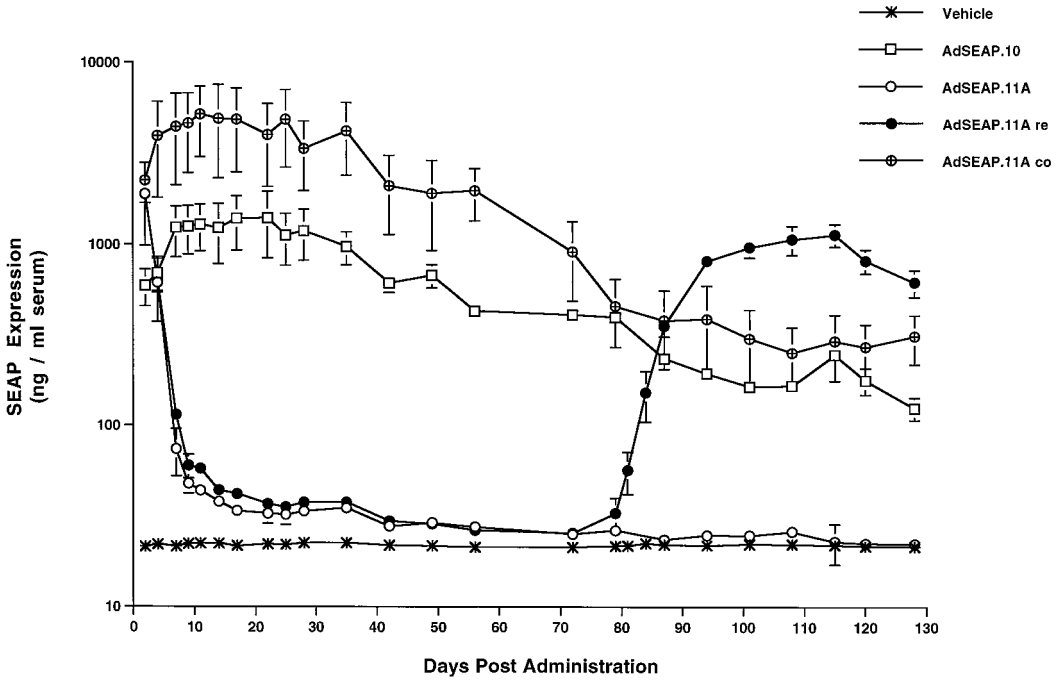

FIG. 8. E4 products activate transgene expression in *trans*. Three animals per group of NIH Swiss nude mice were administered intravenously 10^{11} total particles of each vector: AdSEAP.10, AdSEAP.11A, or AdSEAP.11A plus AdLacZ.10 (AdSEAP.11A co). In addition, three mice were administered 10^{11} total particles of AdSEAP.11A at day 0 and then administered 10^{11} total particles of AdLacZ.10 at 79 days postadministration (AdSEAP.11A re). At the indicated times postadministration, serum was collected, SEAP expression was detected by chemiluminescence, and the average was determined. Error bars indicate standard error of the mean.

Cells in tissue culture do not appear to exhibit this process since vector genomes can be maintained in transduced primary cells for extended periods of time without loss (3a).

Several models can be invoked to explain these results. Crystal and coinvestigators (49, 50) have recently suggested that innate immune mechanisms are involved in substantial clearance of vector and may account for at least a part of this dramatic effect. In addition, other models of viral clearance that do not kill the infected cell have been described (18). These noncytopathic processes are mediated through the expression of effectors like gamma interferon and tumor necrosis factor. Recently Bruder and Kovesdi (5) have shown that binding to cell surface receptors and internalization of vector particles can activate expression of cell effectors that may be influencing this process of viral clearance.

In immunocompetent animals, we have detected that 0.5 to 1% of vector genomes can be retained and persist for extended periods of time. Therefore, persistent transgene expression is possible from these genomes. Understanding the nature and mechanisms involved in this genome persistence in immunocompetent animals will be critical for developing persistent-expression vectors for therapy of chronic diseases.

In immunodeficient animals, the 2 to 5% of vector genome that persists is not static in its ability to express transgene over time. This is best seen in the comparison of PolIII viral expression (VA RNA) and the transgene expression cassettes driven by PolII in our vectors. While VA RNA expression appears to correlate with the level of vector genome, transgene expression from a GV10 genome does not. These studies show that the GV11 genome is not activated for transgene expression like the GV10 genome in the immunodeficient mouse.

An interesting question for future investigation is whether transgene expression cassette placement in different regions within the vector genome will alter its ability to be activated. It is possible that these effects are dependent on how each expression region is sublocalized within the transduced cell nucleus. Future analysis of vectors with modified transgene expression cassette placement will help to uncover the dynamic changes that can occur to the viral chromatin and the transduced cell during and after transduction.

It can be deduced from these studies that the presence of E4 products provides an enhanced cellular environment for transgene expression. Several plausible models could explain how E4 products might be involved in this function. Various E4 gene products have been shown to alter cellular transcriptional potential, and the expression cassettes used in this study may be activated through one of these molecular mechanisms. E4-ORF4 does this by modifying the activity of protein phosphatase 2A (28, 35). E4-ORF6/7 can complex with the cellular transcription factor E2F and alter expression profiles of a number of cellular and viral genes (38). E4-ORF3 may be able to do this by reorganization of cellular or other viral factors within the nucleus (6, 12, 17). Given that several possibilities exist, the molecular basis of this activation mechanism must await the construction and evaluation of vectors that express only individual E4 products and vectors that utilize other promoters and expression cassettes. In this regard, it is interesting that vectors expressing only E4-ORF6 do not activate transgene expression from a transgene expression cassette using a CMV promoter (1, 3a).

The E4 promoter is significantly reduced in activity in the absence of E1A (37), which implies that the E4 products provide a very potent function that may be more catalytic in nature than stoichiometric. Alternatively, the low level of E4 products produced in a transduced cell may start a cascade of events wherein multiple cellular and/or viral modifications lead to the activation of the viral transgene expression cassette. Consistent with this model is the observation that

after readministration, it takes several days for transgene expression from the GV11 vector genome to reach the high level of expression found with GV10. While we have been studying the effects of E4 products on transgene and other viral expression cassettes, it is likely that these products also alter cellular gene expression. Therefore, these E4 products are extremely potent cell modulators and from the perspective of a safer gene transfer vector would be best removed. However, reduced transgene persistence is traded for increased safety in this vector background. Only future evaluation into these mechanisms will provide a vector to accomplish both.

Transgene expression from the RSV and CMV promoter expression cassettes used in these studies is effected by the presence of E4 products. It is clear from this study that the PolIII promoter encoding VA RNA is not altered by the presence of E4 products. The universality of these activation effects has yet to be shown. Armentano and coworkers have shown that expression from an E1A expression cassette does not persist in any vector background tested (1). This finding suggests that like VA RNA in this study, the low-level expression from the E1A expression cassette is not altered by the presence of E4 products. These results support the hypothesis that an activation mechanism is responsible for high-level persistent transgene expression in immunodeficient animals. It is assumed that each promoter and expression cassette will fall in one of the two categories and that it will be necessary to test each for functionality in a variety of contexts. In this study, we have evaluated the E4 activation effects in liver and lung which appear to function similarly. The universality of these expression effects in other tissues has yet to be shown. It is highly likely that not all expression cassettes are further down-regulated by the absence of E4 products as is the CMV expression cassette used in this study. Therefore, the utility of the CMV expression cassette in a GV11 vector backbone is excellent for short-term high-level expression which may be applicable in several acute therapy settings and provide a much safer vector for gene transfer.

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