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Effect of the E4 Region on the Persistence of Transgene Expression from Adenovirus Vectors

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The utility of adenovirus vectors for gene therapy is limited by the transience of expression that has been observed in various in vivo models. Immunological responses to viral targets can eliminate transduced cells and cause the loss of transgene expression. We previously described the characterization of an E4 modified adenovirus, Ad2E4ORF6, which is replication defective in cotton rats. We reasoned that gene transfer vectors based on Ad2E4ORF6 would have a reduced potential for viral gene expression in vivo which might be beneficial for achieving persistence of transgene expression. E1 replacement vectors expressing the cystic fibrosis transmembrane regulator or β-galactosidase were constructed as series of vectors that differed with respect to the E4 region. Vectors containing a wild-type E4 region, E4 open reading frame 6, or a complete E4 deletion were compared in the lungs of BALB/c mice for persistence of expression. Results obtained with nude mice indicate that nonimmunological factors have a major influence on the longevity of transgene expression. Expression was transient from the E1a promoter with all vectors but persisted from the cytomegalovirus promoter only with a vector containing a wild-type E4 region. Transience of expression did not correlate with the disappearance of vector DNA, suggesting that promoter down-regulation may be involved. Coinfection studies indicate an E4 product(s) could be supplied in *trans* to allow persistent expression from the cytomegalovirus promoter. In summary, the choice of promoter is important for achieving persistence of expression; in addition, some promoters are highly influenced by the context of the vector backbone.

Recombinant adenoviruses have been developed as gene transfer vectors for vaccine and gene therapy applications. Several features of adenoviruses have made them attractive candidates for use as gene therapy vectors (4). They can be easily grown and purified in large quantities and can transduce a wide variety of dividing and postmitotic cells. However, the success of in vivo gene transfer with high doses of first-generation vectors has been limited by transient transgene expression and the development of host inflammatory and immune responses. Several reports indicate that cellular immune responses to viral antigens may be a principal factor in the elimination of transduced cells and loss of transgene expression (30, 31, 34). Studies indicating that immunosuppressive agents can prolong transgene expression lend further support to this hypothesis (11, 20). However, in some instances where the process has been examined in more detail, persistence of expression seems to be primarily affected by the immunogenicity of the transgene rather than viral proteins (2, 28).

The replication defect of E1-deleted viruses can be overcome in cultured cells at high multiplicities of infection (6). This may be due, in part, to the presence of cellular factors with E1a-like activity that have the potential to transactivate viral promoters, resulting in vector replication (18, 27). High multiplicities of infection in vivo as well as the presence of host cellular factors with E1a-like activity could lead to the expression of viral antigens. This in turn could elicit a cellular immune response toward transduced cells leading to their elimination and consequent loss of transgene expression. For these reasons, additional changes have been introduced into the backbone of adenovirus vectors to render them more replication defective and, thus, further reduce their potential for viral gene expression. Two regions of adenovirus, E2 and E4, that play critical roles in viral DNA synthesis and late gene expression have been targeted for these changes in the development of second-generation recombinant viruses (1, 10, 15, 29, 32, 33).

The E2a region encodes a single-stranded DNA binding protein that is required for initiation and chain elongation in viral DNA synthesis and is involved in controlling early and late viral RNA metabolism. A temperature-sensitive mutation, *ts*125, and a deletion have been introduced into the E2a region (10, 15, 33). Studies done with CBA mice, cotton rats, and nonhuman primates indicate that transgene expression could be prolonged from vectors containing the E2a *ts*125 mutation (10, 14). In these studies, prolonged transgene expression observed with second-generation vectors correlated with diminished levels of late proteins detected in the tissues examined. Contrary to these reports, a recent study using BALB/C mice and hemophilia B dogs demonstrates that this E2a mutation is insufficient for achieving persistent expression (12).

The E4 region encodes functions required for viral DNA replication, mRNA splicing and accumulation, late protein expression, and inhibition of host cell protein synthesis. We previously described the construction and characterization of an adenovirus, Ad2E4ORF6, which has been modified in the E4 region such that it contains only open reading frame (ORF) 6 (1). Although this virus is replication competent in cultured cells, it is replication defective in cotton rats. We reasoned that second-generation gene transfer vectors containing an E1 deletion and the Ad2E4ORF6 modification might consequently have a reduced potential for viral gene expression in vivo compared to first-generation vectors containing a wild-type E4

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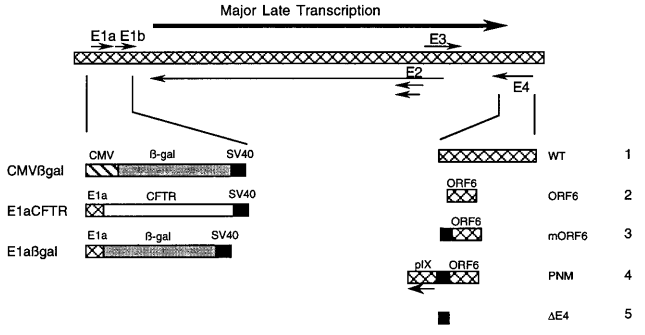

FIG. 1. Genomic structure of recombinant adenovirus vectors. Expression cassettes replacing the E1 region are depicted on the left and variations of the E4 region are depicted on the right. E4 variations are as follows: 1, wild-type (WT) E4 region; 2, Ad2E4ORF6 E4 modification; 3, same as 2 but includes an SV40 polyadenylation signal between the fiber region and ORF6; 4, same as 3 except pIX sequences have been relocated between the fiber region and the SV40 polyadenylation signal; 5, complete E4 deletion (ΔE4). See Materials and Methods for details of construction.

region, and that this might impact cellular immune responses and persistence of transgene expression. E1 replacement vectors containing various β-galactosidase or cystic fibrosis transmembrane regulator (CFTR) expression cassettes were constructed as series of vectors that differed with respect to the E4 region. Persistence of transgene expression was analyzed for each series in the lungs of both parental and nude BALB/c mice. We find that persistent gene expression in the mouse lung is difficult to achieve, even in the absence of a host cellular immune response. Our results are consistent with transcriptional down-regulation as a primary determinant responsible for transience of gene expression. Furthermore, in those specific cases where we can demonstrate long-term expression, our results suggest that it is dependent on both the promoter used to control expression and the context of the E4 region.

MATERIALS AND METHODS

Construction of vector series. The expression cassettes and variations of the E4 region that were used to create vector series are depicted in Fig. 1. The CMVβgal expression cassette was constructed in a pBR322-based plasmid that contained Ad2 nucleotides 1 to 10680 from which nucleotides 357 to 3328 were deleted. The deleted sequences were replaced with a cytomegalovirus (CMV) immediate-early promoter (obtained from pRC/CMV; Invitrogen), a *lacZ* gene encoding β-galactosidase with a nuclear localization signal, and a simian virus 40 (SV40) polyadenylation signal (nucleotides 2533 to 2729). The E1aCFTR and E1aβgal expression cassettes were similarly constructed, except that the Ad2 deletion started from nucleotide 545. The cDNA for CFTR represents nucleotides 123 to 4622 of the published sequence, and the *lacZ* gene was obtained as a *Not*I fragment from pCMVβ (Clontech).

For simplicity, the variations of the E4 region have been assigned numbers. As shown in Fig. 1, a wild-type E4 region is assigned the number 1 and the Ad2E4ORF6 backbone is assigned the number 2. Variation number 3 (mORF6) is identical to number 2 but includes an SV40 polyadenylation signal (nucleotides 2533 to 2729) inserted between the fiber region and ORF6. This modification corrects the reduced fiber synthesis that is observed with Ad2E4ORF6 (unpublished results). Variation number 4 (protein IX moved [PNM]) is based on mORF6 and contains pIX sequences (nucleotides 3519 to 4061) that have been deleted and relocated between the fiber region and the SV40 polyadenylation signal. This modification helps to reduce the generation of replication-competent adenovirus during virus expansion and has been invaluable for generating clinical-grade preparations of virus (16). A complete E4 deletion, variation 5 (ΔE4), was constructed as follows. pAdORF6 (1) was cut with *Bam*HI and *Sal*I, which removes the inverted terminal repeat (ITR) and E4ORF6. This segment was replaced with a *Bam*HI-*Bgl*II fragment containing the SV40 polyadenylation signal and a *Bam*HI-*Sal*I fragment generated by PCR containing Ad nucleotides 35642 to 35937 (E4 enhancer region and ITR).

Some previously described vectors have been assigned different names for simplicity. The vectors CMVβgal-1 and -2 refer to Ad2/βgal-2 and Ad2/βgal-4, respectively (19), and the vector E1aCFTR-4 refers to Ad2/CFTR-8 (17).

Viruses and cell lines. All viruses, except those with complete E4 deletions, were propagated in 293 cells and purified and titered by end point dilution with fluorescein isothiocyanate-conjugated anti-hexon antibody (Chemicon) as previously described (1). Viruses with complete E4 deletions were propagated in VK2-20 cells, which is an E4-complementing cell line derived from 293 cells (22). Both 293 and VK2-20 cells were obtained from Frank Graham.

In vivo delivery of recombinant viruses to mouse respiratory epithelium. BALB/c parental and nude mice were purchased from Taconic Farms. Animals, mostly females, ranging from 7 to 16 weeks old were used for in vivo studies. Mice were anesthetized by inhalation of Metofane (methoxyflurane) and were infected by intranasal instillation of 3×10^9 infectious units (i.u.) of recombinant virus in 100 μl of phosphate-buffered saline–3% sucrose.

β-Galactosidase assay. Lungs from individual animals were homogenized, and β-galactosidase activity was measured by a 3-{4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo(3,3,1,1^{3,7})decan]-yl}phenyl β-D-galactopyranoside (AMPGD assay) (Galactolight; Tropix). The protein concentration in lung homogenates was measured with the Bio-Rad DC reagent, and β-galactosidase activity is expressed as relative light units (RLU) per microgram of protein.

DNA extraction and analysis. DNA was extracted by homogenizing lung tissue in 600 μl of lysis buffer for 90 s in a Mini-Bead beater (Biospec Corp.) before adding 30 μl of proteinase K (10 mg/ml) and incubating at 42°C for 2 h. Equal aliquots of supernatant for each animal in a group were pooled. DNA was then precipitated by adding 7.7 M ammonium acetate to reach a 2 to 2.5 M final concentration and 2 volumes of 100% ethanol and incubating on ice. Nucleic acid was resuspended in diethyl pyrocarbonate-treated water.

DNA samples from animals that received the CMVβgal series were subjected to Southern analysis. Fifteen micrograms of DNA was cut with *Afl*II and electrophoresed through a 0.6% agarose gel. DNA was transferred to GeneScreen Plus and hybridized to a probe to detect β-galactosidase sequences. After three washes at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and three washes at 65°C in 0.2× SSC–0.1% SDS, the filter was dried and autoradiographed. DNA samples from animals that received the E1aβgal series were similarly treated except that 25 μg of DNA was cut with *Bln*I and electrophoresed on an 0.85% agarose gel and the filter was analyzed on a PhosphorImager for quantification of hybridization signals.

DNA samples from animals that received the E1aCFTR series were subjected to PCR analysis which was run in duplicate for each group at each time point. DNA was amplified with 2.5 U of *Taq* polymerase (Perkin-Elmer) in a mixture containing final concentrations of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.75 mM $MgCl_2$; 0.01% gelatin; 200 mM (each) dTTP, dATP, and dGTP; 20 mM dCTP; 500 nM (each) primers CF3111+ and CF3620−; and 0.5 ml of [^{33}P]dCTP (3,000 Ci/mmol) for 30 cycles. Amplified products were separated by electrophoresis through a 2% agarose gel and stained with ethidium bromide. The gel was dried, analyzed on a PhosphorImager (Molecular Dynamics), and quantitated by using image analysis software (ImageQuant).

RNA extraction and reverse transcription (RT)-PCR. Tissue samples from each mouse were homogenized in RNA Stat-60 solution (Tel-Test B, Inc.). RNA was extracted according to the acid guanidinium thiocyanate-phenol-chloroform method (7) in a Mini-Bead beater. RNA samples were pooled for animals in each group per time point.

Total RNA (7.5 mg) was subjected to DNase treatment. In addition to the sample RNA, 10^3 or 10^2 molecules of synthetic competitive RNA (a 469-bp chloramphenicol acetyltransferase transcript with CFTR sequences at both ends) was added to the reaction mix. RT was performed with a cDNA kit from Invitrogen. Following RT, cDNA from each test group was amplified by using the conditions and reagent concentrations stated above for the PCR. The PCR product from the hCFTR sequence is 544 bp; the competitor product is 469 bp. The ratio of the intensity of the products was compared to those of a DNA standard curve containing log concentrations of Ad2/CFTR-8 DNA (from 10^1 to 10^6) run with the appropriate number of copies of competitor DNA to quantitate gene expression.

Isolation and culture of rat hepatocytes. Inbred Brown-Norway rat hepatocytes were isolated by collagenase digestion (5) and were infected in suspension by cold incubation for 1 h with recombinant adenoviruses at a multiplicity of infection (MOI) of 10. The cells were cultured in the Biocoat HP differentiation environment. This is an integrated system optimized to support cultures of differentiated hepatocytes in a serum-free in vitro environment for up to 30 days. The cultures were fed every 3 days and kept at 37°C in an atmosphere of 5% CO_2.

RESULTS

Effect of E4 on persistence of expression in vectors containing the CMV promoter. Based on the characteristics of Ad2E4ORF6 (1), it was predicted that vectors containing this modification would have less potential to replicate in vivo compared to vectors containing a wild-type E4 region and that this might consequently lead to better profiles of transgene expression. Vectors were constructed that varied only in the E4 region and were tested in BALB/c mice to determine the effects the ORF6 modification might have on persistence of expression. Initially, a comparison was made between

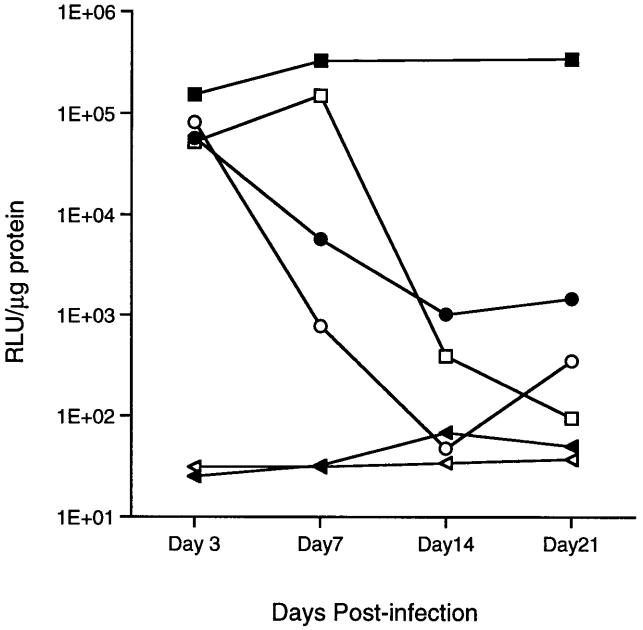

FIG. 2. β-Galactosidase expression in the lungs of BALB/c mice that received CMVβgal-1 or -2. Both parental and nude mice were infected by intranasal instillation of 3×10^9 i.u. of either virus. Mice were sacrificed at various time points postinstillation, and the β-galactosidase activity was measured in the lung tissue. Each time point represents the average from three animals for CMVβgal-1 (nudes, ■; parentals, □) and CMVβgal-2 (nudes, ●; parentals, ○), except for vehicle controls, which are averages from two animals (nudes, ◀; parentals, ◁).

CMVβgal-1 (wild-type [wt] E4) and CMVβgal-2 (ORF6). As shown in Fig. 2, expression of β-galactosidase declined in BALB/c mice that received either vector. This was expected and is most likely due to the immunogenicity of β-galactosidase in immune-competent animals. The decline in expression, however, was more precipitous in animals that received CMVβgal-2. In BALB/C nude mice, expression persisted to day 21 in CMVβgal-1-treated animals but declined from day 3 in CMVβgal-2-treated animals. The rapid loss of expression in both immune-competent and nude mice suggests that, contrary to our expectations, the ORF6 modification does not have a beneficial effect on the persistence of expression. The difference in expression between CMVβgal-1 and CMVβgal-2 in the absence of T- and B-cell responses suggested that nonimmunologic factors are involved in persistence and result from the change in the E4 region. The increase in β-galactosidase expression between day 3 and day 7 in both parental and nude mice that received CMVβgal-1 most likely represents an accumulation of enzyme and can be accounted for by its half-life (measured in cultured cells as being approximately 14 days) (26a).

From the CMVβgal-1 and -2 comparison, it is unclear whether the wild-type E4 region in CMVβgal-1 is required for and/or positively influences persistence of expression or whether ORF6 in CMVβgal-2 is detrimental. One possible explanation considered for the observed results is that the vectors may differ in cytotoxicity. It has well been established that the E1 region of adenovirus encodes regulators of apoptosis which act in both p53-dependent and p53-independent mechanisms. A recent report has indicated that the p53-independent mechanism involves early region 4 (24). It also has been shown that ORF6 binds to p53 and can block p53-mediated transcriptional activation. Since p53 can act as a tumor suppressor by inducing growth arrest or apoptosis, it is possible that an E4ORF6-p53 interaction could lead to induction of apoptosis in infected cells (8). Because E4 gene products may be involved in both p53-dependent and -independent apoptotic pathways, CMVβgal-1 and -2 were compared for their ability to induce apoptosis. Primary human fibroblasts and A549 cells were infected with each of the viruses at MOIs of 100 and 1,000 and were examined by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling method (13). This method uses terminal transferase to incorporate dUTP-biotin at the free ends of fragmented DNA which is then detected by immunohistochemistry. Results (data not shown) indicated that there was no difference in the number of apoptotic nuclei in cultures infected with CMVβgal-1 or -2, which suggests that there is no difference in apoptotic potential between the two vectors. This suggests that the difference in expression observed between CMVβgal-1 and -2 in nude mice might not be due to clearance (loss) of vector-infected cells by apoptotic cell death.

Although nude mice are deficient in B cells and T cells, they produce macrophages that can release tumor necrosis factor alpha (TNF-α). It has been shown that the expression of adenovirus E1a in various mouse cell lines renders them susceptible to TNF-α-mediated cytolysis. We next tested the hypothesis that ORF6 expression might have an effect on mouse cells similar to that of E1a. C127 cells were infected with either CMVβgal-1 or -2 and were exposed to increasing concentrations of TNF-α. Cytolysis was measured as lactate dehydrogenase released into the medium. By this assay, no sensitivity to cytolysis by TNF-α was observed in C127 cells infected with either CMVβgal-1 or -2 (data not shown). This suggests that the disappearance of β-galactosidase expression observed in nude mice that received CMVβgal-2 was most likely not due to clearance of cells by TNF-α-mediated cytolysis.

A simpler approach for investigating differences between CMVβgal-1 and -2 was to construct a vector containing a complete E4 deletion, CMVβgal-5, and to compare the expression profile of this vector to that of CMVβgal-1 and CMVβgal-2. As seen in Fig. 3A, the expression of β-galactosidase in nude mice that received CMVβgal-5 parallels that which is observed for CMVβgal-2. Because this decline in expression occurs in the absence of any contribution from the E4 region, a requirement or positive influence of a wild-type E4 region on persistence of expression seems likely. Vector DNA levels were also measured in these animals by Southern analysis, and as shown in Fig. 3B, comparable levels were detected in the day 14 samples of all three vectors. Therefore, the decline in expression observed in CMVβgal-2- and CMVβgal-5-infected animals cannot be accounted for by a loss of vector DNA. Because the DNA of all three vectors persists but expression differs, down-regulation of expression from the CMV promoter in the absence of a wild-type E4 region seems likely and suggests a positive regulatory role of E4 in persistence of expression.

Effect of E4 on the persistence of expression in vectors that contain the E1a promoter. The results obtained with the CMVβgal series indicated that a wild-type E4 region is required to achieve persistence of expression. We next wished to determine if E4 would similarly affect persistence of expression from another promoter. The results obtained with an E1aβgal series are shown in Fig. 4A. In this series, which comprises E1aβgal-1 (wild-type E4), E1aβgal-2 (ORF6), and E1aβgal-3 (mORF6), the adenovirus E1a promoter is used to drive transgene expression. Analysis of β-galactosidase expression in nude mice indicates that the E4 region has little impact on the

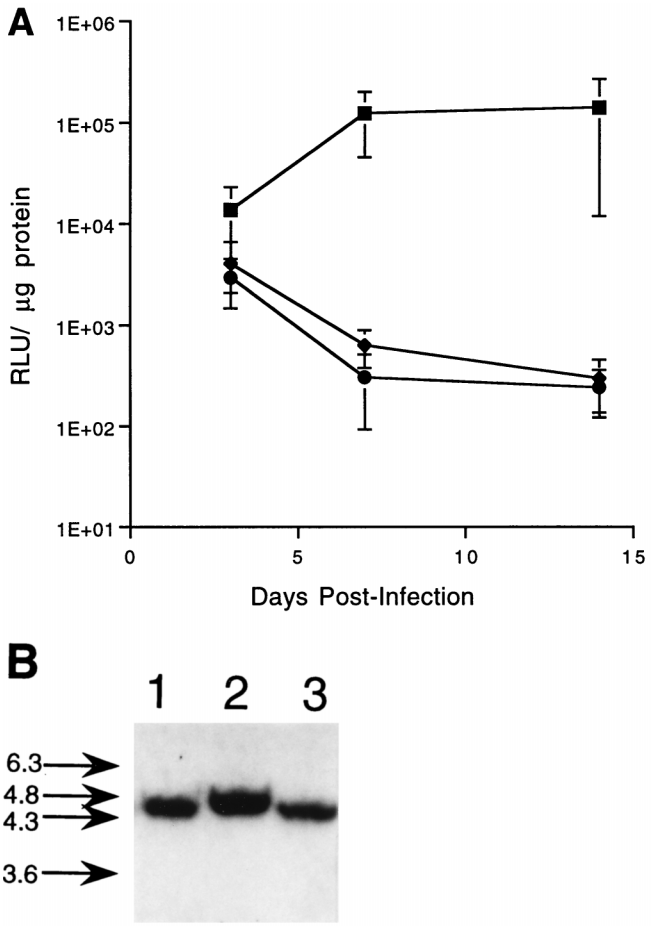

FIG. 3. (A) β-Galactosidase expression in mice that received CMVβgal-1, -2, or -5. Nude mice were infected by intranasal instillation of 3×10^9 i.u. of each virus. Mice were sacrificed on days 3, 7, and 14 postinstillation, and the β-galactosidase activity was measured in the lung tissue. Each time point represents the average from four animals for CMVβgal-1 (■), CMVβgal-2 (●), and CMVβgal-5 (◆). (B) Southern analysis. DNA was isolated from lung tissue and was restricted with *Afl*II, which yields a 4.4-kbp fragment containing the β-galactosidase expression cassette in all three vectors. DNAs from the day 14 time points were subjected to Southern analysis with a probe to detect β-galactosidase sequences in lungs that received CMVβgal-2 (lane 1), CMVβgal-1 (lane 2), or CMVβgal-5 (lane 3). Arrows on the left indicate the positions of lambda *Bst*EII marker fragments (sizes are in kilobase pairs).

persistence of expression. While expression may be somewhat higher with E1aβgal-1 initially, by day 43 expression declines by two orders of magnitude to background levels with all three vectors. Because transient expression is observed in the absence of B- and T-cell responses, other nonimmunologic mechanisms appear to influence persistence. The presence of vector DNA in the lungs was also analyzed to determine if the loss of expression could be correlated with loss of vector DNA. Southern analysis indicates that vector DNA levels are maintained in nude mice at approximately 30% of day 3 levels for all three vectors at both the 14- and 43-day time points (Fig. 4B). This indicates that the decline in expression of approximately two orders of magnitude cannot be completely accounted for by a loss of vector DNA and that other factors that regulate expression are probably involved.

In order to rule out possible effects of the transgene on persistence of expression, a vector series containing CFTR instead of βgal was also analyzed. The E1aCFTR series comprises E1aCFTR-1 (wild-type E4), E1aCFTR-4 (PNM), and E1aCFTR-5 (ΔE4). The profile of expression obtained in nude mice with this series is shown in Fig. 5A. Expression, measured as copies of CFTR mRNA, drops two orders of magnitude by day 14 irrespectively of the E4 region used in the vector. The drop in vector DNA levels to approximately 10% of day 3 levels, as measured by PCR (Fig. 5b), does not appear to account for the decline in CFTR mRNA. The results obtained with this vector series are similar to those obtained with the E1aβgal series, suggesting that the E1a promoter may not be a suitable choice when persistent gene expression in the murine lung is the desired endpoint. Furthermore, in contrast to the results described above for the CMVβgal series, the context of the E4 region has little or no impact on persistence in vectors where the E1a promoter is used to control transgene expression.

***trans* effect of E4 on persistence of expression.** The results obtained with the CMVβgal series indicate that a wild-type E4 region is required for persistence of expression. However, the results obtained with both the E1aβgal and the E1aCFTR series indicate that expression is independent of the context of the E4 region and does not persist with the E1a promoter. One explanation of this difference might be that a gene product(s) of E4 activates or relieves repression of the CMV promoter yet has no effect on the E1a promoter. Another possibility is that the strong enhancer of the CMV promoter is acting distally on viral promoters, including E4, which may allow persistence. To investigate this further we tested whether the effect of E4 could be supplied in *trans* from a vector that contains a wild-type E4 region but does not contain a CMV promoter. For this purpose, nude mice were infected with CMVβgal-1 or CMVβgal-2 alone or were coinfected with E1aCFTR-1 (wild-type E4), E1aCFTR-4 (PNM), or E1aCFTR-5 (ΔE4). If a wild-type E4 is required for persistence then coinfection of CMVβgal-1 (wild-type E4) with any of the E1aCFTR series should not alter the expression profile and persistence of expression should be observed in all cases. If E4 is required but its effect can be supplied in *trans* then coinfection of CMVβgal-2 (ORF6) with E1aCFTR-1 (wild-type E4) should result in persistence of expression similar to that which is observed with CMVβgal-1. In contrast, coinfection with either E1aCFTR-4 (PNM) or -5 (ΔE4) should not effect expression, and an expression profile similar to that observed in animals that received CMVβgal-2 alone would be expected. The results shown in Fig. 6 indicate that the expression profile from CMVβgal-1 (bar A) remains unchanged in animals that were coinfected with any of the E1a-CFTR vectors (bars B, C, and D). Expression from CMVβgal-2 (bar E) drops by day 14 in animals that were coinfected with E1aCFTR-4 or -5 (bars G and H). However, expression on day 14 in animals that were coinfected E1aCFTR-1 (bar F) remains elevated and is similar to that seen in animals that received CMVβgal-1 (bars A to D). This suggests that an E4 product(s) is supplied in *trans* and can act, either directly or indirectly, to allow persistence of expression from the CMV promoter.

A similar coinfection study was performed with cultured rat hepatocytes to determine if an E4 requirement for expression from the CMV promoter would be observed in other tissues. Rat hepatocytes were infected with CMVβgal-1 or -2 alone or were coinfected with E1aCFTR-1, -4, or -5 and were analyzed for β-galactosidase expression by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. Fig. 7A and B depicts β-galactosidase expression observed with CMVβgal-1 on days 3 and 14 postinfection, respectively. Panels C, D, and E correspond to expression observed 14 days postinfection in cultures that were coinfected with E1aCFTR-4, -1 and -5, respectively. In all cases, expression seems to persist to day 14. Panels

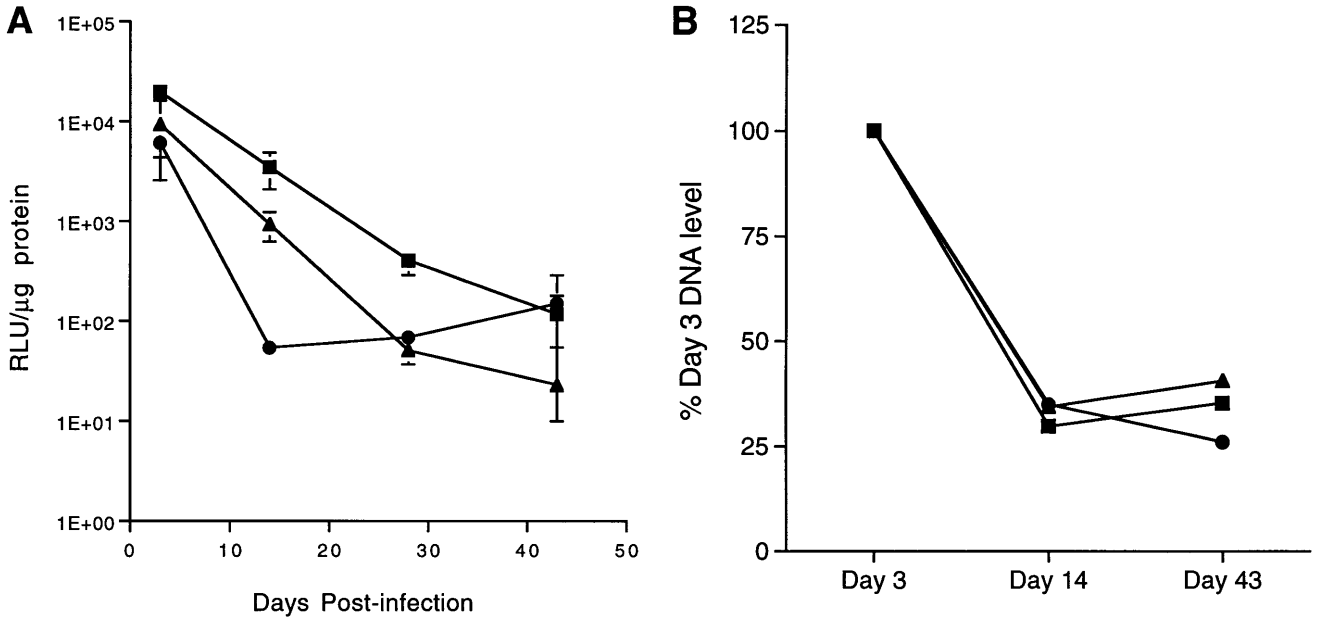

FIG. 4. (A) β-Galactosidase expression in mice that received the E1aβgal series. BALB/c mice infected by intranasal instillation of 3×10^9 i.u. of virus were sacrificed on days 3, 14, and 43 postinstillation. β-Galactosidase activity was measured in the lungs of mice that received E1aβgal-1 (■), E1aβgal-2 (●), or E1aβgal-3 (▲). Each time point represents the average activity in the lungs of three animals. (B) DNA analysis. DNA was isolated from lungs at the indicated time points and was subjected to Southern analysis for detection of β-galactosidase sequences. Hybridization signals were quantitated by PhosphorImager analysis, and the results are plotted as percentages of the day 3 level for E1aβgal-1 (■), E1aβgal-2 (●), and E1aβgal-3 (▲).

A and B in Fig. 8 represent CMVβgal-2-infected cultures analyzed on days 3 and 14 postinfection, respectively. Expression with this vector in hepatocytes has clearly diminished during this time period. Coinfection with E1aCFTR-4, -1, and -5, shown in panels C, D, and E, indicates that expression declines by day 14, except in the culture that was coinfected with E1aCFTR-1. These results demonstrate a requirement for E4 for prolonged expression from the CMV promoter in cultured hepatocytes, as was the case in the mouse lung.

DISCUSSION

Initial in vivo studies with first-generation adenovirus vectors have indicated that transgene expression is transient and

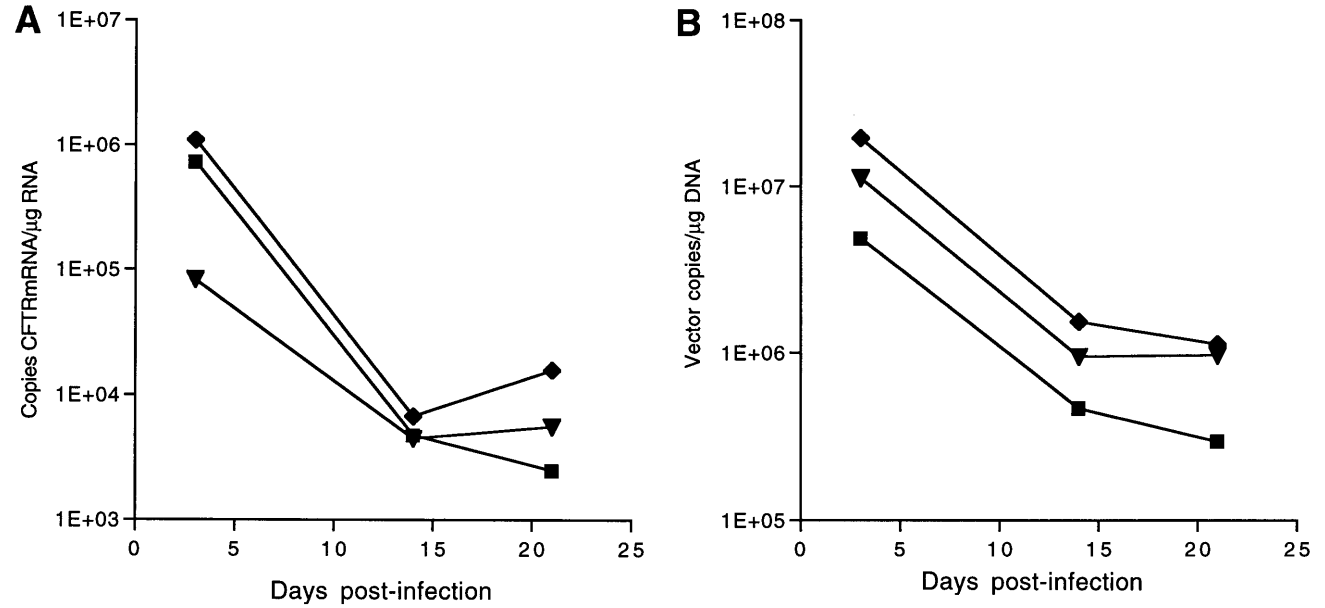

FIG. 5. (A) Expression of hCFTR mRNA in BALB/c nude mice following intranasal instillation of vectors in the E1aCFTR series. Mice received 3×10^9 i.u. of E1aCFTR-1 (■) or E1aCFTR-4 (▼) or 4.8×10^8 i.u. of E1aCFTR-5 (◆) and were sacrificed on days 3, 14, and 21 postinstillation. RNA samples from lungs were pooled at each time point ($n = 3$) with each vector, and hCFTR mRNA was measured by quantitative RT-PCR. (B) DNA analysis. DNA samples from lungs were pooled at each time point, and the presence of vector DNA was analyzed by PCR to detect hCFTR sequences in animals that received E1aCFTR-1 (■), E1aCFTR-4 (▼), or E1aCFTR-5 (◆).

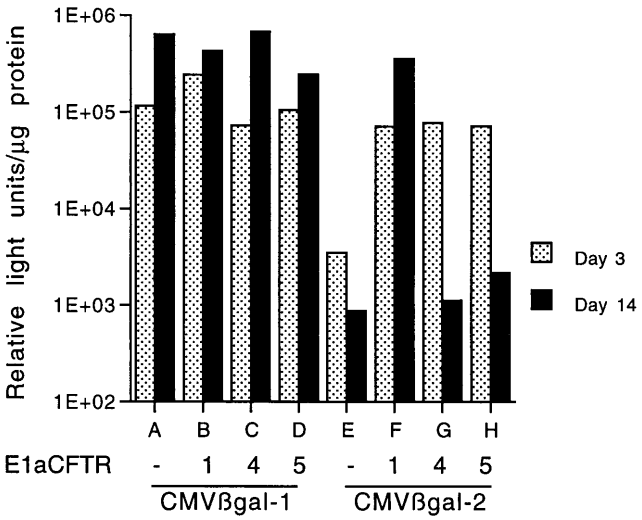

FIG. 6. Effect of E4 on β-galactosidase expression in the lungs of nude mice. Mice were infected by intranasal instillation of 1.5×10^9 i.u. of CMVβgal-1 (bars A to D) or CMVβgal-2 (bars E to H) either alone (bars A and E) or with 1.5×10^9 i.u. of E1aCFTR-1 (bars B and F), 1.5×10^9 of i.u. E1aCFTR-4 (bars C and G), or 4.3×10^8 i.u. of E1aCFTR-5 (bars D and H). Mice were sacrificed on days 3 and 14, and β-galactosidase activity was measured in lung homogenates. Each bar represents the average activity from at least three animals.

that this is probably due, in part, to the expression of viral genes in target cells that elicit cellular immune responses and clearance of the vector-infected cells. It has been proposed that second-generation vectors that are doubly defective might reduce the occurrence of viral gene expression and that this might allow for improved persistence of transgene expression. Some success has been reported with second-generation viruses that are E1 deleted and have a temperature-sensitive mutation in E2a.

We previously described the construction and characterization of second-generation adenovirus gene transfer vectors that have been modified in the E4 region to retain only ORF6 of E4 (1). While the deleted ORFs are dispensable for virus growth in cultured cells, experiments with cotton rats showed that they appear to be required for virus replication in vivo. We reasoned that second-generation vectors containing this modification would be additionally replication defective compared to first-generation vectors and that this might lead to improved persistence of expression. Series of expression vectors were constructed that varied in the E4 region and were tested for profiles of expression in both parental and nude BALB/c mice. Results obtained with nude mice with the CMVβgal series were unexpected and indicated that, in the absence of B- and T-cell responses, the E4 region seemed to have a regulatory role in expression from the CMV promoter. Persistence of expression was observed only with a vector that had a wild-type E4 region and not with vectors that contained ORF6 or a complete E4 deletion. A wild-type E4 region (or perhaps an ORF) seems to be required for persistence of expression when the CMV promoter is used to drive transgene expression in the murine lung.

Although we have not yet ascertained the mechanism underlying our observations, there are several plausible models which we are investigating. The E4 region is involved in transcriptional regulation and viral DNA replication during the adenovirus life cycle. It is possible that gene products of the E4 region may affect expression within a vector either by acting directly or indirectly, in this case on the CMV promoter, or by allowing a low level of vector replication that may be required for persistence of expression. The E4 ORF4 gene product, for example, is reported to mediate the activity of protein phosphatase 2A and thus the phosphorylation state of various transcription factors (21, 26). It is possible that E4 ORF4 can affect transcription factors that may be required for the maintenance of expression from the CMV promoter. The ORF6/7 gene product complexes with transcription factor E2f and is required for maximal accumulation of E2a mRNA (25). Although there are apparently no E2f binding sites in the CMV promoter, an indirect effect of ORF6/7 expression on the CMV promoter may be involved. Initially, it also seemed possible that overexpression of E4 ORF6 from certain of our vectors could be interfering with regulation of the CMV promoter by p53. However, this explanation seems unlikely in that expression did not persist even with a vector containing a complete E4 deletion.

The E4 ORF3 gene product, alone, functions in the relocalization of cellular factors into viral replication domains in the nucleus (9). It is possible that the reorganization of these factors in the nucleus affects vector expression or allows a low level of vector replication which may be required for persistence of expression. It has recently been suggested that vector replication is required to achieve persistence of expression in the liver (23). Our results, however, are more consistent with promoter down-regulation as the primary factor determining transience of expression. We have demonstrated that vector DNA appears to persist in the lung irrespective of the specific E4 modification, yet expression profiles differ. Improved persistence has been reported with vectors that are further crippled for DNA replication by a temperature-sensitive mutation in E2a. In addition, expression persists from the CMV promoter in AdβgalΔE1E2 (33), a vector in which E2a has been deleted (26b). These findings are inconsistent with a hypothesis of conventional vector DNA replication as an explanation for our observed results since E2a is absolutely required for viral DNA synthesis. This, however, does not rule out a low level of nonconventional vector replication by cellular enzymes that may be aided by the E4 region.

Our results with the CMV promoter differ from results obtained with the E1a promoter. In both the E1aβgal and E1aCFTR series the context of the E4 region had little or no impact on the persistence of expression. Expression did not persist with any vector that utilized the E1a promoter to drive transgene expression. Our results with the human phosphoglycerate kinase promoter in AdCFTR-2 (19), while perhaps not optimal, suggest that this promoter is less affected by E4 and may be active in the absence of a complete E4 region. We have also analyzed other enhancers/promoters for the ability to produce long-term gene expression in the mouse lung. Preliminary analysis of expression from the cytokeratin 18, cytokeratin 14, or hybrid CMV/E1a promoter did not demonstrate persistence in vectors containing the E4ORF6 modification, although we do not yet know if they will behave differently in the context of a vector backbone which retains a wild-type E4 region (26b).

Coinfection studies in the lung and in cultured hepatocytes indicate that an E4 product(s) can act in *trans* on the CMV promoter and prevent expression from being down-regulated. These results suggest that the lung and liver may contain factors which down-regulate or repress expression from the CMV promoter. This is consistent with a recent study in which tissue-specific expression was analyzed in transgenic mice containing the *lacZ* gene regulated by the CMV promoter (3). Expression was reported in a variety of tissues which correlated well with tissues naturally infected by human cytomegalovirus in hu-

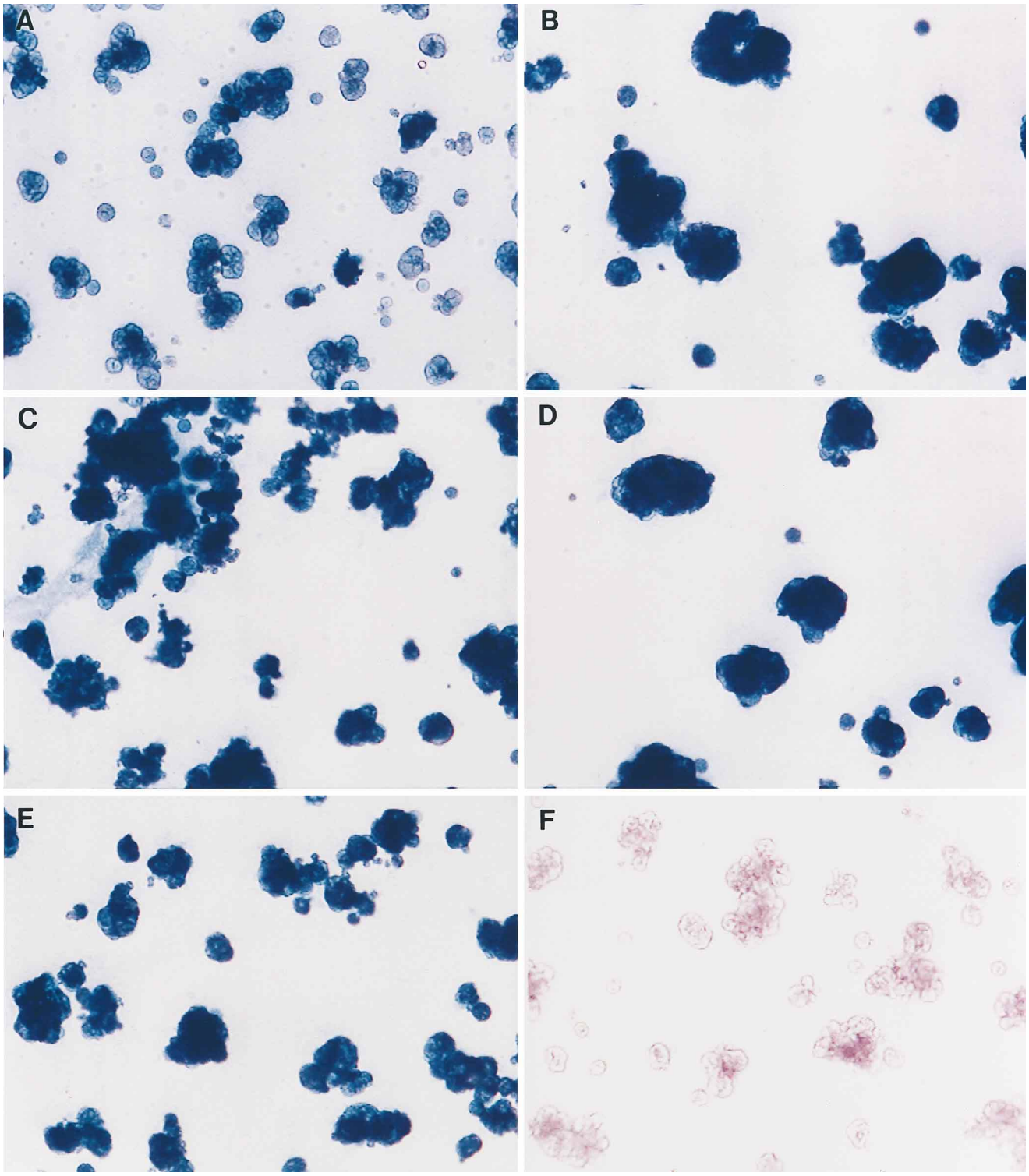

FIG. 7. Rat hepatocytes infected with CMVβgal-1. Rat hepatocytes were infected with CMVβgal-1 alone at an MOI of 10 or were coinfected at an MOI of 10 with vectors in the E1aCFTR series. β-Galactosidase expression was visualized by X-Gal staining. Panels A and B represent cultures stained on days 3 and 14 postinfection, respectively, with CMVβgal-1 alone. Panels C, D, and E represent cultures stained 14 days postcoinfection with E1aCFTR-4, -1, and -5, respectively. Panel F is an uninfected, stained culture.

mans. Expression, however, was not detected in the lung or liver, which are not tissues commonly associated with human cytomegalovirus infection. This suggests that other factors are required for CMV promoter activity in these tissues. In support of this hypothesis, a recent study of adenovirus vectors which examined the suitability of various promoters for hepatic gene therapy, found that the CMV promoter yielded high-level but transient expression in the livers of immune-deficient mice

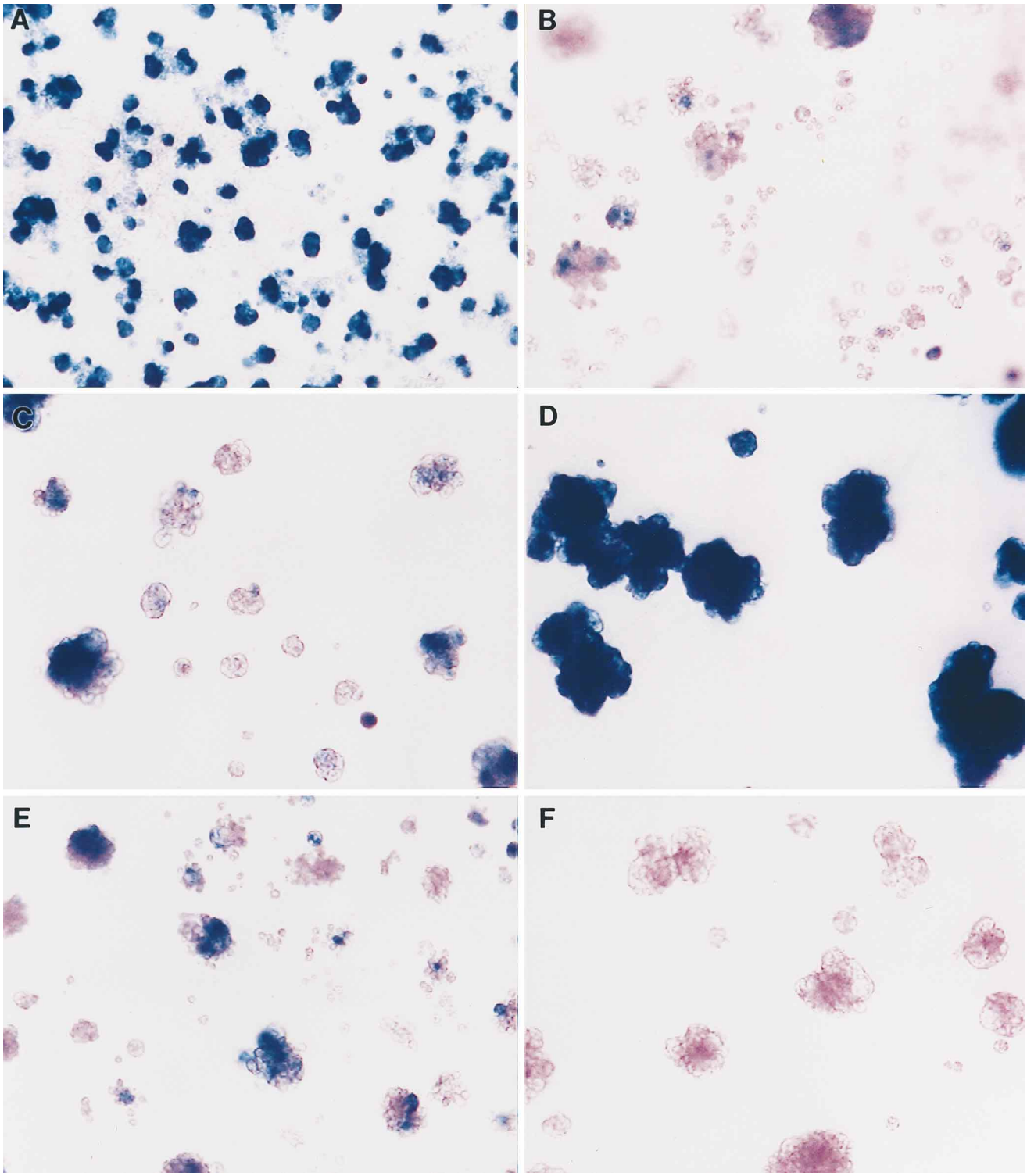

FIG. 8. Rat hepatocytes infected with CMVβgal-2. Rat hepatocytes were infected with CMVβgal-2 alone at an MOI of 10 or were coinfected at an MOI of 10 with vectors in the E1aCFTR series. β-Galactosidase expression was visualized by X-Gal staining. Panels A and B represent cultures stained on days 3 and 14 postinfection, respectively, with CMVβgal-2 alone. Panels C, D, and E represent cultures stained 14 days postcoinfection with E1aCFTR-4, -1, and -5, respectively. Panel F is an uninfected, stained culture.

(16). Interestingly, transient expression was observed in this model despite the presence of a wild-type E4 region in the vector, a result which is not consistent with our observations on isolated rat hepatocytes. It is possible that differences in results are due to species differences. Nonetheless, further studies will have to be done to determine if a requirement for an E4 factor is limited to the CMV promoter and to various murine cell types.

It has been suggested that the E4 region be deleted from adenovirus vectors since ORF6 binds to p53 and can interfere with transcription (8). Coinfection results presented here indicate that an E4 factor(s) other than ORF6 can act in *trans* (either directly or indirectly) on the CMV promoter. This raises the possibility that E4 factors may similarly act on cellular promoters and alter gene regulation. The interaction of E4 proteins with host factors could have negative, neutral, or even positive effects upon the efficacy of gene therapy. However, assessment of the impact that the deletion or retention of E4 ORFs has on prolonging transgene expression will require using a promoter that can achieve persistent expression independently of any regulatory effects imposed by the E4 region.

Much emphasis has been placed on elucidating immunological factors that limit persistence of transgene expression from adenovirus vectors. Indeed, it is now clear that cellular and humoral immune responses to both the transgene and the adenoviral vector can limit gene transfer and expression. However, the data presented above provide compelling evidence that the choice of vector backbone and transgene transcription unit can also profoundly influence longevity of gene expression in the absence of an acquired immune response. How broadly this principle will apply to other vector modifications, target tissues, and species is not yet known. In the absence of a detailed understanding of the mechanisms underlying molecular persistence, future generations of adenoviral vectors should first be assessed in immune-deficient animals before examining their properties in more relevant in vivo models.

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