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A Gene Transfer Vector-Cell Line System for Complete Functional Complementation of Adenovirus Early Regions E1 and E4

DOUGLAS E. BROUGH,* ALENA LIZONOVA, CHI HSU, VICTORIA A. KULESA, AND IMRE KOVESDI

GenVec Inc., Rockville, Maryland 20852

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The improvements to adenovirus necessary for an optimal gene transfer vector include the removal of virus gene expression in transduced cells, increased transgene capacity, complete replication incompetence, and elimination of replication-competent virus that can be produced during the growth of first-generation adenovirus vectors. To achieve these aims, we have developed a vector-cell line system for complete functional complementation of both adenovirus early region 1 (E1) and E4. A library of cell lines that efficiently complement both E1 and E4 was constructed by transforming 293 cells with an inducible E4-ORF6 expression cassette. These 293-ORF6 cell lines were used to construct and propagate viruses with E1 and E4 deleted. While the construction and propagation of AdRSVβgal.11 (an $E1^-/E4^-$ vector engineered to contain a deletion of the entire E4 coding region) were possible in 293-ORF6 cells, the yield of purified virus was depressed approximately 30-fold compared with that of $E1^-$ vectors. The debilitation in AdRSVβgal.11 vector growth was found to correlate with reduced fiber protein and mRNA accumulation. AdCFTR.11A, a modified $E1^-/E4^-$ vector with a spacer sequence placed between late region 5 and the right inverted terminal repeat, efficiently expressed fiber and grew with the same kinetic profile and virus yield as did $E1^-$ vectors. Moreover, purified AdCFTR.11A yields were equivalent to $E1^-$ vector levels. Since no overlapping sequences exist in the E4 regions of $E1^-/E4^-$ vectors and 293-ORF6 cell lines, replication-competent virus cannot be generated by homologous recombination. In addition, these second-generation $E1^-/E4^-$ vectors have increased transgene capacity and have been rendered virus replication incompetent outside of the new complementing cell lines.

Adenovirus vectors are promising tools for in vivo gene transfer. These viruses can infect a wide variety of cell types and tissues, can effect gene transfer and expression of the transgene at a high level in quiescent and proliferating cells, and can be grown to high titers in complementing cell lines. Early versions of adenovirus gene transfer vectors were based on early region 1 (E1) and/or E3 deletions. Subsequent versions of adenovirus vectors have disabled E2A (7) or E4 (24, 42, 44) in addition to E1 or E3. By altering the expression of these virally encoded regions, the gene transfer vector is expected to show increased transgene persistence and decreased inflammatory responses because of the reduction in the number of viral antigens.

The adenovirus E4 constitutes ~10% of the viral genome, and genetic analysis has demonstrated that E4 products are essential for productive virus infection. A complex set of phenotypes has been demonstrated by deletion analysis of E4, showing that E4 products play vital roles in viral infection. E4 proteins are involved in several levels of regulation of cellular and viral gene expression, viral DNA replication, late viral RNA accumulation, viral protein synthesis, host shutoff, virus assembly, E2 expression, and adeno-associated virus helper function (9, 13, 36, 43). Several sets of differentially spliced mRNAs are generated from the E4 region during viral infection (6, 8, 40, 41). Of the possible open reading frame (ORF) products encoded by the E4 region, only one, either ORF3 or ORF6, is absolutely required for viral growth in tissue culture (2, 10, 13, 18).

The regulatory proteins of E4 interact and antagonize cellular and viral targets. ORF3 and ORF6 have been shown to control late viral mRNAs at a posttranscriptional level by increasing the nuclear stability of pre-mRNAs transcribed from the major late promoter (29, 30, 32, 37). The interaction of ORF6 with the E1B 55-kDa protein leads to the inhibition of p53-mediated transcriptional activation and to selective cytoplasmic accumulation of mRNA (33, 45). E4 products are also involved in controlling the crucial cellular transcription factor E2F in adenovirus-infected cells (25). The E4-ORF4 gene product is involved in controlling the phosphorylation status of cellular and viral proteins (19, 27). In addition, a transforming function has been linked to E4-ORF1 from human adenovirus type 9 (Ad9) (15–17).

The removal of these viral products that modulate transduced cells in the subsequent generations of gene transfer vectors is highly desirable. We have constructed a vector-cell line system in which adenoviruses with deletions in both E1 and E4 can be dually complemented, easily constructed, and grown to the same high levels as first-generation adenovirus vectors. The new 293-based complementing cell lines were engineered to express a single E4 product, ORF6, from the inducible metallothionein promoter. Since no overlapping sequences between the new cell lines and the genome of the vector with E4 deleted exist, no generation of replication-competent virus by homologous recombination will occur. The deletion of both E1 and E4 further cripples virus replication in transduced cells. The deletion of E1, E3, and E4 in these transfer vectors allows for increased transgene capacity to 10 kb. Moreover, by using E1, E3, and E4 regions for transgene placement, a single adenovirus vector can be used for multitransgene expression.

Construction of 293-ORF6 cell lines. To functionally complement for E4, the Ad5 E4-ORF6 gene (Ad5 bp 33190 to 34084) was cloned by PCR with specific primers containing flanking *Pac*I restriction enzyme sites into the metal-inducible metallothionein promoter expression cassette plasmid pMT-

* Corresponding author. Mailing address: GenVec Inc., 12111 Parklawn Dr., Rockville, MD 20852. Phone: (301) 816-0396, ext. 200. Fax: (301) 816-0440.

TABLE 1. Inducible expression of E4-ORF6 in 293-ORF6 cell lines

Cell line[a]	ORF6 expression[b]
A2	+++
A21	(+)
A22	(+)
A23	−
A24	(+)
A25	(+)
A26	−
A27	(+)
A28	−
A29	(+)
A210	−
A211	−
A212	+
A213	−
A214	+++++
A224	−
A231	+
A232	++
A237	++
A261	+++
A259	−
B8	++
B81	(+)
B82	++
B83	+
B84	+
B85	(+)
B86	−
B87	+
B88	++
B89	++
B810	(+)
B814	(+)
B816	−
B818	−
B819	−
B820	−
B821	−
B822	−
B823	−
B824	−
B827	−

[a] Cells were grown in the presence of puromycin to maintain selection.
[b] Established monolayers from independent clonal isolates were induced with 100 μM $ZnCl_2$ for 24 h, and the expression of the ORF6 gene was detected by Northern (RNA) blotting. The relative levels of expression from lowest [(+)] to highest (+++++) are noted. −, no expression.

puro (GenVec Inc., Rockville, Md.) to generate pMT-E4-ORF6. 293 cells were transfected with *Sca*I-linearized pMT-E4-ORF6, and transformed cells were selected for growth in the presence of puromycin (Puro^r). Selection for Puro^r included the addition of 0.5 μg of puromycin (Sigma Chemical Co., St. Louis, Mo.) per ml to the growth medium (Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum, 100 μg of streptomycin per ml, and 100 μg of penicillin per ml; all components were from Life Technologies, Gaithersburg, Md.). Transformation, propagation, and isolation of transformants were done as previously described for dominant selectable markers (3, 21). Colonies remaining after 2 weeks of growth under selection conditions were isolated and passaged for 8 weeks to ensure stability with respect to Puro^r. A total of 42 independent cell lines were established.

The 42 Puro^r cell lines were screened for the ability to express E4-ORF6 (ORF6$^+$). Northern mRNA analysis (Table 1) showed that 24 of the 42 Puro^r cell lines (57%) also inducibly expressed E4-ORF6. Ten of the ORF6$^+$ cell lines expressed only low levels, 13 expressed moderate levels, and 1 expressed high levels of E4-ORF6. The moderate- to high-level E4-ORF6-expressing cell lines were screened for the ability to complement deletion virus growth. All of the 293-ORF6 cell lines retained the ability to functionally complement the E1$^-$/E4^{wt} virus AdRSVβgal.10. However, these 293-ORF6 cell lines complemented the growth of E1^{wt}/E4$^-$ virus H5dl366 to various levels and this complementation did not directly correlate with the level of induced ORF6 expression. The removal of these 293-ORF6 cell lines from selection conditions for more than 10 months has not changed their ability to complement the growth of E1$^-$, E4$^-$, or E1$^-$/E4$^-$ virus.

Construction of E1$^-$/E4$^-$ virus. To determine the utility of these 293-ORF6 cell lines for the construction and propagation of E1$^-$/E4$^-$ vectors, a complete deletion of E4 was engineered in the E1$^-$ virus AdRSVβgal.10. Virus construction relied on a single in vivo recombination within the overlapping regions between left and right fragments of the genome (39). The E1$^-$/E4$^-$ virus AdRSVβgal.11 (Fig. 1) was constructed by transfecting 293-ORF6 cells with the left genome fragment, 0 to 75 map units (m.u.) of *Srf*I and *Spe*I restriction enzyme-digested viral DNA, from AdRSVβgal.10 DNA and a right genome fragment provided from a plasmid containing adenovirus m.u. 59.5 to 100 (*Bam*HI and *Sal*I restriction enzyme digested) into which a full deletion of the E4 coding sequence had been introduced. This plasmid, pGBSΔE4, is a version of a pGem-based plasmid (Promega, Madison, Wis.) that contains Ad5 m.u. 59.5 to 100 cloned at respective *Bam*HI-to-*Sal*I restriction sites, pGBS (GenVec Inc.). To construct the E4 deletion (Ad5 bp 32830 to 35566) in pGBS, a *Pac*I site was generated at Ad5 bp 32830. A *Pac*I-*Sal*I fragment that encodes Ad5 bp 35564 to 35935 was prepared by PCR and cloned into the respective restriction sites to generate pGBSΔE4. AdRSVβgal.11 plaque isolates were screened by PCR for the presence of the E4 deletion with primers that spanned Ad5 bp 32792 to 35879. The purity of the virus stock after two sequential plaque isolations was confirmed by PCR and restriction enzyme analysis of viral DNA. Virus plaque isolation, propagation, and analysis of growth properties were done as previously described (3, 4, 20, 21), with the modification that the final quantitation of virus yield was determined on 293-ORF6 cell lines by either PFU, focus-forming units (FFU) with a polyclonal antibody directed against the entire adenovirus capsid, or FFU with a monoclonal antibody directed against the adenovirus DNA-binding protein (5). Viral DNA was isolated by digesting infected-cell lysates or purified virus with proteinase K in the presence of sodium dodecyl sulfate.

AdRSVβgal.11 characterization. The production of purified AdRSVβgal.11 was approximately 30-fold depressed compared with that of AdRSVβgal.10 (Table 2). To understand the reason for this debilitation, virus growth characterization of AdRSVβgal.11 was performed. The kinetics of AdRSVβgal.10 and AdRSVβgal.11 growth on 293 cells (data not shown) and the 293-ORF6 cell line A2 (see Fig. 4) were compared. The growth of AdRSVβgal.10 on either 293 or A2 cells was the same. AdRSVβgal.11 virus growth and the expression of late capsid protein were detected only in 293-ORF6 cell lines, not in infected 293, HeLa, or A549 cells (data not shown). AdRSVβgal.11 growth was delayed in infected 293-ORF6 cells, and the plateau of virus yield was significantly reduced compared with that of AdRSVβgal.10 and did not increase even up to 7 days postinfection.

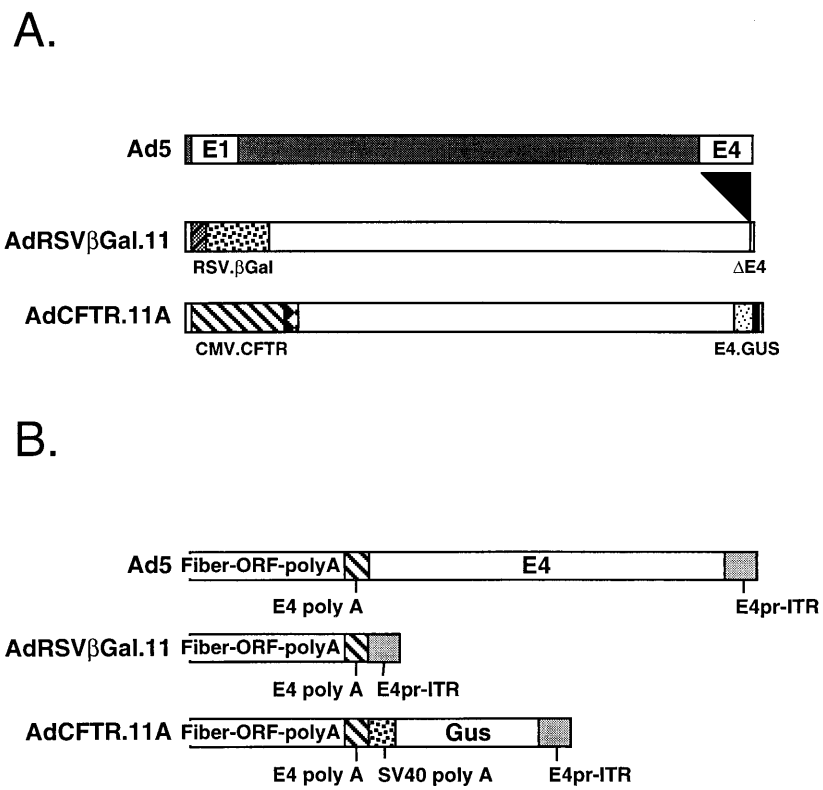

FIG. 1. (A) Schematics of E1$^-$/E4$^-$ viruses. Only the pertinent E1 and E4 regions are represented. (B) Schematics of E4 regions. SV40, simian virus 40; E4pr, E4 promoter.

The delay and reduction in virus growth in AdRSVβgal.11-infected 293-ORF6 cells were not due to reduced viral DNA replication. Figure 2A shows similar levels of viral DNA accumulation in AdRSVβgal.10- and AdRSVβgal.11-infected A2 cells at 24 h postinfection. In addition, the AdRSVβgal.10 and AdRSVβgal.11 DNA synthesis levels, as determined by the incorporation of [^{3}H]thymidine at 16 h postinfection, were similar (data not shown). These results suggest that 293-ORF6 cell lines provide all the E1 and E4 functions necessary for DNA replication of E1$^-$/E4$^-$ viruses.

The reduction in AdRSVβgal.11 virus production correlated with a reduction in the accumulation of the late protein fiber (Fig. 2B and Fig. 3). The methods used for protein evaluation have been previously described (1, 3, 4). While the level of fiber accumulation was depressed, the amount of fiber found on purified AdRSVβgal.11 capsids was not affected (data not shown). Fiber was the only major capsid protein whose levels

TABLE 2. Purified-vector production yields[a]

Vector	Cell line	Active particles (FFU)/cell[b]
AdRSVβgal.10	293	615
AdCFTR.10	293	650
AdRSVβgal.11	A2	22
AdCFTR.11A	A2	720

[a] Vectors were purified from infected cells at 2 or 3 days postinfection by three freeze-thaw cycles followed by three successive bandings on cesium chloride gradients. The purified vector was dialyzed in 10 mM Tris (pH 7.8)–150 mM NaCl–10 mM $MgCl_2$–3% sucrose, aliquoted, and stored at −80°C.

[b] Active particle determinations were performed on 293-ORF6 cells with an anti-DNA-binding protein monoclonal antibody.

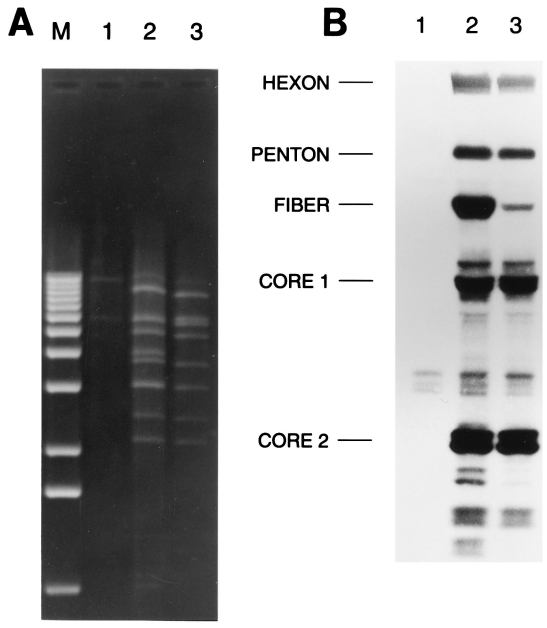

FIG. 2. (A) Viral DNA replication in AdRSVβgal.10- and AdRSVβgal.11-infected A2 cells. A2 cells were infected at a multiplicity of infection of 10. Viral DNA accumulations were compared by harvesting low-molecular-weight DNA (12, 35) from infected cells at 24 h postinfection and digesting an equal fraction of each DNA preparation with *Kpn*I before electrophoresis on a 0.8% agarose gel. Lane M, size markers; lane 1, mock-infected cells; lane 2, AdRSVβgal.10-infected cells; lane 3, AdRSVβgal.11-infected cells. (B) Viral late protein expression in A2 cells. At 36 h postinfection at a multiplicity of infection of 5, cells were harvested and the level of late protein accumulation was detected by immunoblotting using a polyclonal antibody directed against the adenovirus capsid. Lane 1, mock-infected cells; lane 2, AdRSVβgal.10-infected cells; lane 3, AdRSVβgal.11-infected cells.

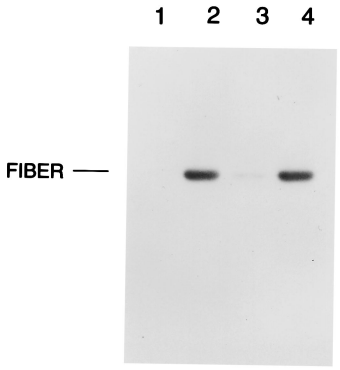

FIG. 3. Fiber protein accumulation in A2 cells. A2 cells were infected at a multiplicity of infection of 5. At 20 h postinfection, protein was extracted and the amount of fiber was detected by immunoblotting using a polyclonal antibody directed against fiber. Lane 1, mock-infected cells; lane 2, AdRSVβgal.10-infected cells; lane 3, AdRSVβgal.11-infected cells; lane 4, AdCFTR.11A-infected cells.

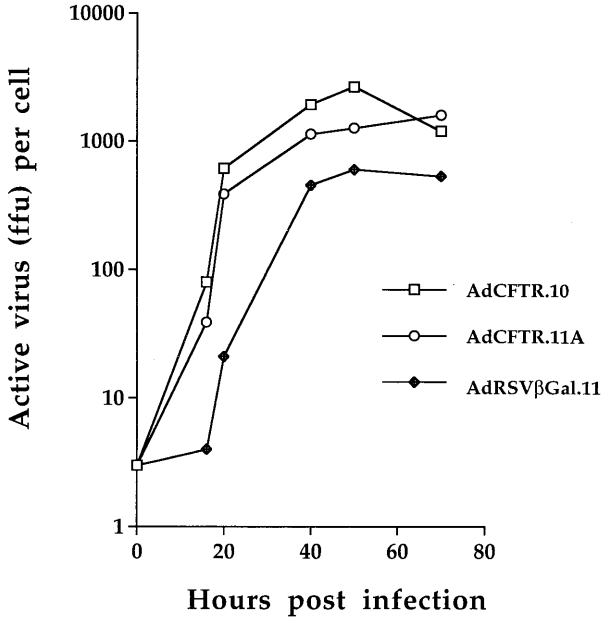

FIG. 4. Kinetics of AdCFTR.10, AdCFTR.11A, and AdRSVβgal.11 virus production. Infected A2 cells were harvested at the indicated times postinfection, and the amounts of virus in cell lysates (in FFU) were determined.

were reduced in AdRSVβgal.11-infected A2 cells, and its lower accumulation correlated with a reduction in virus growth. However, as can be seen in Fig. 2B, the level of the minor capsid protein pVIII was also reduced in AdRSVβgal.11-infected A2 cells. The level of pVIII reduction did not directly correlate with a reduction in virus growth. While mature pVIII is critical in the formation of capsids (38), the importance of its reduction in AdRSVβgal.11-infected A2 cells is not known. The reduction in fiber protein accumulation directly correlated with reduced cytoplasmic accumulation of late region 5 (L5) mRNA (data not shown). These results suggested that the deficiency in virus production was due to altered L5 mRNA expression. Taken together, the results implied that either the 293-ORF6 cell lines were deficient in a key function necessary for proper L5 mRNA expression or that the E4 deletion in AdRSVβgal.11 somehow altered L5 mRNA expression.

Construction and characterization of AdCFTR.11A. If the E4 deletion in AdRSVβgal.11 altered L5 mRNA expression, the debilitation was most likely caused by destabilization of the L5 mRNA. To test this hypothesis, an expression cassette with a strong stop and polyadenylation signal was placed within the deleted E4 region, which spaced the L5 region from the right inverted terminal repeat (ITR).

AdCFTR.11A (Fig. 1) was constructed by cotransfecting the left genome fragment (0 to 90 m.u.) from AdCFTR.ORF6 (2a) and the linearized transfer plasmid pNSE4Gus. To facilitate manipulation of the E4 region, Ad5 bp 31089 to 35935 (*Nde*I-*Sal*I fragment) was subcloned from pGBSΔE4 into pUC19 to generate pNSΔE4. pNS11A was constructed by introducing first a polylinker region (*Sma*I, *Kpn*I, and *Eag*I sites) between the *Mun*I and *Pac*I sites of pNSΔE4 and then an *Eag*I-*Sma*I fragment from pAdCMVβGlu (GenVec Inc.) that contained the glucoronidase gene (*gus*) and the simian virus 40 polyadenylation signal between the fiber and E4 polyadenylation stop sites and the ITR. Plaques of AdCFTR.11A appeared within 10 days and were stained blue by the addition of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) substrate to the medium. Secondary plaques of AdCFTR.11A were purified and analyzed to ensure the purity of the virus stock as described above for AdRSVβgal.11.

The levels of fiber protein accumulation for AdRSVβgal.10, AdRSVβgal.11, and AdCFTR.11A were compared in 293-ORF6 cells (Fig. 3) to determine if the new E4 deletion construct in AdCFTR.11A corrected the defect in L5 expression. The amount of fiber accumulation in AdCFTR.11A-infected A2 cells was equal to that obtained with the wild-type E4 virus AdRSVβgal.10, while the amount of fiber accumulation remained low, as expected, in AdRSVβgal.11-infected cells. The removal of all of the E4 coding sequences in AdRSVβgal.11 appears to be the reason for the reduction in the cytoplasmic mRNA levels of L5. It is feasible that a critical L5 mRNA signal was deleted, but since no known L5 signal exists within the sequences removed, this is unlikely. It is more probable that the juxtaposition of the ITR and E4 promoter elements next to the L5 region generated the destabilization of the L5 message. The correction of L5 mRNA expression in AdCFTR.11A-infected cells by the inclusion of a transcriptional cassette between the ITR and L5 supports this idea. There are several possible mechanisms for L5 mRNA destabilization. These include possible important distance or spacing requirements for efficient L5 expression, improper termination of the L5 message, or the generation of antisense L5 RNA from the E4 promoter. Additional experiments are under way to decipher the mechanism.

The kinetics of AdCFTR.11A virus growth in A2 cells mirrored that of AdCFTR.10 (Fig. 4). In addition, the production of triple cesium chloride gradient-purified AdCFTR.11A virus in A2 cells was at a level similar to that seen with first-generation vectors in 293 cells (Table 2). The ratios of total particles to active particles obtained with AdCFTR.11A preparations were in ranges similar to those obtained with first-generation viruses (20 to 40 total particles per FFU).

The results indicate that these 293-ORF6 cell lines provide all the necessary E1 and E4 functions and confirm that E4-ORF6 is sufficient for full complementation of E4 function. It is interesting that E4-ORF6/7 is not required for full E4 complementation, given the key role E4-ORF6/7 plays in E2 transactivation (14, 26, 28, 31, 34) through the cellular transcription factor E2F (22, 23). This observation is in agreement with those of Hemström et al. (11) and suggests that full activation of the E2 promoter by E4-ORF6/7 is not required

for virus growth in tissue culture. While it is possible that full activation of the E2 promoter can be accomplished through other mechanisms in these 293-ORF6 cell lines, it is interesting to speculate that the activation of E2F via E4-ORF6/7 and the subsequent activation of the E2 promoter may play an important function for virus growth in vivo.

The vector-cell line system described here provides the necessary tools for the construction and high-level production of $E1^-/E4^-$ vectors. We have built a library of E1/E4-complementing cell lines that have proven to be extremely useful in the construction of $E1^-/E4^-$ vectors. By using this vector-cell line system purified $E1^-/E4^-$ virus can be produced at $E1^-$ vector levels. Moreover, we have shown a system in which E4 alterations can be designed, cloned, and moved into virus by a similar methodology and with similar efficiency as are first-generation $E1^-$ vectors. In addition, the design and construction of AdCFTR.11A show the potential of using multiple transgenes housed in different regions within the same vector.

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