Adenovirus 35 Vectors with Improved Transgene Capacity, and Ease of Production

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Abstract

Adenovirus 35 (Ad35) based vectors are now in clinical testing. Here we present improvements to the present Ad35 based vectors. We developed an integrated Ad35 vector platform comprising vectors with expanded transgene capacity, and ease of production on an FDA approved manufacturing cell line (293-ORF6). Presently Ad35 vectors rely on deletion of the essential E1 region as the mechanism to generate replication-deficient vectors. By creating E1-E3- vectors we and others have expanded the space available for transgenes. We have further extended the regions that can be deleted by removing the entire E4 region giving an additional 2400 bp of space. This expanded capacity when combined with the E1-E3- deletions will increase the utility of Ad35 vectors by allowing the use of larger genes or alternative regulatory sequences. The removal of E4 also introduces an additional block to virus growth. This was confirmed by our E1-, E1(wt)E4- and E1-E4- vectors being defective for growth on non-complementing A549 cells. The Ad35 platform is also an integration between the production cell line and vector genome design. The 293-ORF6 cell line contains the E1 region and E4 ORF6 gene from Ad5 and complements not only E1-E4- Ad5 but also E1-E4- Ad35 vectors. The deletion of the two essential regions eliminates any possibility of generating replication competent adenovirus (RCA) by homologous recombination since there is no E4 region homology between the vector and cell line. We have further expanded the versatility of the Ad35 platform by modifying its capsid for altered tropism (1) and generation of a 293-ORF6 cell line capable of repressing expression of the transgene during advector production (2, 3). The use of 293-ORF6 cells, which supports growth of non-species C virus, to rapidly improve Ad35 vectors is a paradigm to develop additional vector serotypes.

Abstracts:

1. Modification of the Ad35 fiber to ablate CD46 binding. Einfeld *et. al.*

2. Single Administration of an Adenovirus Vectored Vaccine Provides Fast-Acting Protection Against Foot and Mouth Disease Virus in Cattle. Brough *et. al.*

3. Adenovirus Complementing Cell Line for the Construction of Vectors with Inhibitory Transgenes. McVey *et. al.*

Vector Configurations

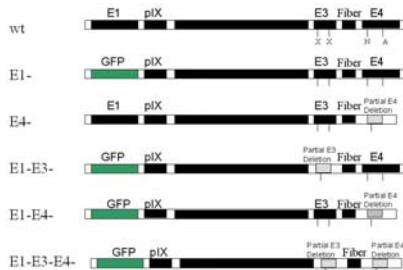


Figure 1. Vector Configurations. Schematic of the various Adenovirus 35 (Ad35) vectors used in these studies is shown. On the wild type (wt) genome the relative location of early region 1 (E1), pIX, early region 3 (E3), Fiber and early region 4 (E4) are designated. A green florescent protein (GFP) expression cassette replaces Ad35 sequences 447-2,916 in the E1 deleted (E1-) vectors. This removes all E1A and most E1B sequences. The deletion of an XbaI restriction fragment (sequences 27,239 to 29,725) was used to generate a partial E3 deletion (E3-). The fusion of AflII and NruI restiction sites was used to create a partial E4 deletion (E4-) of sequences 32,012 to 33,091. This deletion removes all Ad35 E4(ORF6) as well as some adjacent sequences. The restriction sites are represented on the wt genome as X = XbaI, A = AflII and N = NruI. The stippled boxes represent the deleted E3 and E4 regions. The nucleotide numbering system is that of Ad35 Holden sequence.

The Ad35 vectors were created by homologous recombination in *E.coli* using GenVec technology developed for Ad5. The viral genomes were transfected into GenVec's 293-ORF6 complementing cells to generate Ad35 vectors.

E1E4 Complementing Cell Line for Multiple Ad Serotypes

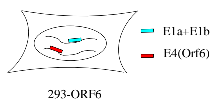


Figure 2. 293-ORF6 Cells Complement Multiple Ad Serotypes. 293-ORF6 cells are used for manufacturing multiple Ad5 E1-E3-E4- vectors for investigational drugs and vaccine candidates that are currently in clinical trials, and are also compatible for the manufacture of Ad35 vectors. These cells are derived from HEK293 cells which express Ad5 E1 (E1a and E1b) and are engineered to also express Ad5 E4(ORF6) genes. The interaction between the E1 and E4 gene products is required for both E1 and E4 complementing activity. The Ad5 E1 and E4 interaction can complement these same functions of Ad35 as well as Ad7 (Nan *et. al. Gene Ther.* 2003 Feb; 10(4)) serotypes. HEK293 cells which contain Ad5 E1 but no E4 sequences complement neither Ad35 E1- nor E1-E4- vectors.

Vector Design Eliminates Possibility of RCA by Homologous Recombination

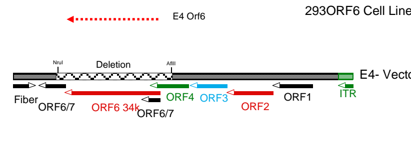


Figure 3. RCA Free Vector Design. A potential contaminant during Ad vector production is the generation of Replication Competent Adenovirus (**RCA**). RCA occurs by recombination of viral sequences (E1 and E4) in the cell line into the viral vector. The RCA is no longer dependent on the complementing cell line for its propagation.

The E4- vector contains a deletion (boxed wavy lines) that removes all E4 ORF6 sequence. This eliminates the possibility of homologous recombination between the E4 ORF6 sequences in the cell line (dashed red arrow) and the vector to generate an RCA. This vector design ensures no RCA can be generated by homologous recombination.

The E4 Region is Essential for AdVector Growth

Virus yield on A549 cells (MOI = 1, 72 hpi)

Virus	Yield (FFU / cell)
1. wt	540
2. E4 –	13

Figure 4. The E4 Deletion Removes an Essential Region. Non-complementing A549 human lung carcinoma cells were infected with a multiplicity of infection (moi) of 1. 72 hours post infection (hpi) virus was harvested. Functional virus yield was determined on 293-ORF6 cells using an FFU assay with a hexon antiserum. The E4- vector shows a greater than 40 fold growth deficit compared to the wild type virus on A549 cells. The E1 deletion likewise removes an essential region of the virus (data not shown). Vectors with the E1 and E4 deletions contain 2 blocks to vector production.

E4- Vector Maintains Fitness

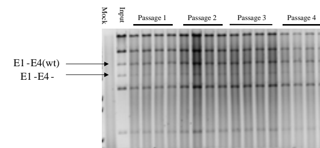


Figure 5. E4- Vector Maintains Fitness. A competition between the E1- and E1-E4- vectors was carried out to determine if the E4 deletion reduced the relative fitness of Ad35 vectors. 293-ORF6 cells were infected in quadruplicate with a moi=1 of both vectors. 72hrs post infections cells were freeze thawed and the lysate used to infect 293-ORF6 cells. This was repeated 3 times. Total DNA was purified, restricted and resolved on an agarose gel to distinguish the two vectors. The location of the diagnostic bands are indicated at the left of the gel. Mock are uninfected cells. Input is the mixture of virus used in the initial infection.

The proportion of E1- and E1-E4- vector is maintained from passage 1 through passage 4. If the E4 deletion introduced a strong growth disadvantage the E1-E4- vector would not be present by passage 4. The E4 deletion removes an essential region of the virus without affecting vector fitness.

pIX Transcription is Maintained with E1, E3, and E4 Deletions

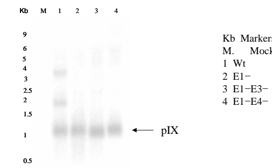
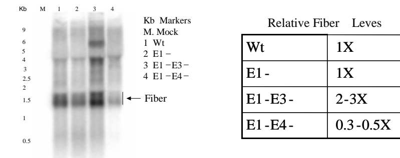


Figure 6. Vector Design Maintains pIX Expression. Loss of pIX expression is reported to cause genetic instability of Ad35 vectors (Havenga et.al. J. Gen. Virol. 2006 Aug; 87(Pt8)). We investigated the effects of the E1, E3 and E4 deletions on pIX transcription by Northern blot analysis. 293-ORF6 cells were infected with a moi=5, harvested 24 hour later, 5 ug of total RNA resolved on an agarose gel, and probed for pIX. The location of the pIX transcript is indicated.

The level of pIX transcripts is the same between the wt virus and the E1-, E1-E3-, and E1-E4- vectors. The E1, E3 and E4 deletions do not affect pIX expression. Interestingly there are two transcripts in the wt virus (~2 and ~3.5 kb) that disappear in the E1- vector. They may represent alternative E1B transcripts that use the pIX polyadenylation signal as is seen in Ad5. Retaining pIX expression should increase genetic stability of the vectors.

E3 and E4 Deletions Alter Fiber Transcription



Relative Fiber Leves

Wt	1X
E1 -	1X
E1 -E3 -	2-3X
E1 -E4 -	0.3 -0.5X

Figure 7. E3 and E4 Deletions Alter Fiber Transcription. The effect of the E1, E3 and E4 deletions was investigated for their effect on levels of fiber expression by Northern blot analysis. 293-ORF6 cells were infected with a moi = 5, harvested 24 hour later, 5 ug of total RNA resolved on an agarose gel, and probed for fiber. The location of the fiber transcript is indicated. There is not a unique but a group of transcripts. Quantitation of relative fiber levels compared to the wt virus is shown in the table at the right.

The three deletions have different effects on fiber transcription. The E1 deletion does not affect fiber expression as expected. The E3 and E4 deletions which flank fiber have opposite effects on fiber levels. The E3 deletion increases while the E4 deletion decreases fiber expression levels to about the same extent. A new band of 6kb appears with the E3 deletion suggesting a change in either splicing or use of polyadenylation sites. This suggests care must be taken when designing E3- and E4- vectors.

Ad35 Vectors With Deletions of Two Essential Regions Have High Productivity

Vector yield (MOI = 1, 72 hpi)

Virus	Yield (PU / cell)
1. E1 –	8.75e4
2. E1 –E3–	5.7e4
3. E1 – E4 –	5.7e4

Figure 8. Good Productivity of E1-, E3- and E4- Vectors. An essential component for gene therapy is the ability to produce the drug. Productivity of the viral vector is also an indication of its robustness. The vectors were used to infect 293-ORF6 cells at a moi=1 and harvested 72 hours post infection (hpi). Total particles (PU) per cell from lysate were determined by HPLC using an Ad35 standard curve.

The E1, E3 and E4 deleted vectors all had yields of over 50,000 pu/cell. This is similar to the productivity of Ad5 vectors. The alternate levels of fiber expression found for these vectors (figure 7) were not deleterious to high vector yields. These three vector designs are productive enough for manufacturing purposes.

Genetic Stability To Ten Passages

Genetic Stability screening (PCR assay with flanking primer paris)

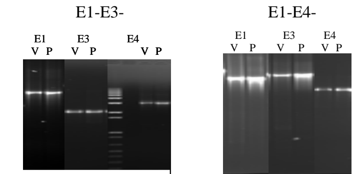


Figure 9. The E1-E3- and E1-E4- Vectors are Genetically Stable Through Ten Passages. Expansion of vector stocks is required for manufacturing purposes. The genetic integrity of the vector needs to be maintained during this process. To model this expansion vector genomes from plasmids were transfected onto 293-ORF6 complementing cell lines. The resulting virus was then passaged ten times. To assess genetic stability, PCRs using primers that flanked the E1, E3 and E4 regions respectively were carried out using purified total DNA from the virus lysate as the template. The E1-E3- and E1-E4- vectors as well as the E1, E3 and E4 regions are indicated. V and P represent the virus and positive control plasmid used as template.

Serial passage of E1-E3- and E1-E4- vectors with genome integrity indicates they can be expanded extensively and are good candidates for GMP manufacturing

Increased Capacity with an E1-E3-E4- Vector

E1 Δ =	2470	bp
E3 Δ =	2480	bp
E4 Δ =	1080	bp
Total =	6030	bp

Figure 10. Increased Carrying Capacity. To expand the utility of Ad35 vectors the carrying capacity of the vector was expanded by combining the E1, E3 and E4 deletions into a single vector. We confirmed the feasibility of this design by constructing the E1-E3-E4- GFP vector (figure 1). An additional six vectors have been constructed with this design. The E1-E3-E4- vector design provides the opportunity to test vector configurations where space is presently limiting.

Summary

- Multiple deletions in Ad35 result in an improved vector system
  - No RCA possible by homologous recombination.
  - Two viral blocks (E1-,E4-) to vector production.
  - Expanded capacity with E1-E3-E4- vectors.
  - GMP manufacturing under development.
    - Vector genomes stable through 10 passages
    - Vector yields of greater than 50,000 pu/cell
  - Vector manufacturing cell line (293-ORF6) available
- The technology to make Ad35 vectors is transferable to other Ad serotypes.