

Adenovirus Complementing Cell Line for the Construction of Vectors with Inhibitory Transgenes

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Abstract

When expressed at high level certain transgenes can impact the construction and production of adenovirus vectors. Reducing expression often allows construction of most of these vectors; however, the lower expression levels may reduce the effectiveness of these vectors for certain applications particularly as vaccines. To overcome this issue, we have generated a complementing cell line (M2A) capable of repressing transcription of the transgenes under the direction of a highly efficient promoter. To make the M2A cell line, 293-ORF6 cells were transfected with a linearized plasmid that encodes the bacterial tetracycline transcriptional repressor (TetR) and hygromycin resistance genes. The base 293-ORF6 cell line is FDA approved for manufacturing E1-E3-E4- vectors. Fifty-nine hygromycin resistant clonal isolates were expanded and screened for expression and activity of TetR. The best isolate identified in the initial screen also showed stable TetR protein expression and DNA copy number even after 38 continuous passages in the absence of drug selection. This cell line represses transcription 10 fold, measured by reverse transcriptase qPCR, from the strong CMV promoter containing a Tet operator positioned between the TATA box and transcription initiation site. Repression is relieved by the TetR antagonist Doxycyclin, and occurs through out the vector growth cycle on these cells. The M2A cell line is capable of supporting the generation of vectors that were previously impossible to construct on 293-ORF6 cells. An example of this is a vector (AdSer1) expressing an antigen from the Foot and Mouth virus (1). This vector was attempted 6 times with no success on 293-ORF6 cells. With M2A cells the virus was readily made on 3 of 3 attempts. The ability to make vectors with inhibitory genes will expand the investigation and application of a number of interesting genes not only for vaccine but also for therapeutic purposes.

1) Abstract # 753: Vectored Vaccine Provides Fast-Acting Protection Against Foot and Mouth Disease Virus in Cattle. Brough et al.

How Tet Repression Works

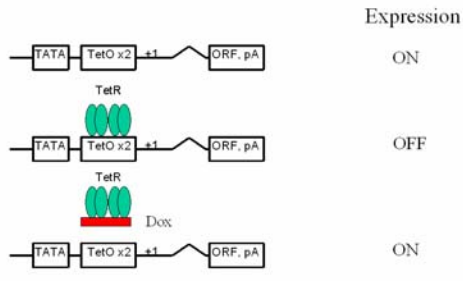


Figure 1. Repression of Transcription by Tetracycline Repressor (TetR). TetR is a bacterial transcriptional repressor that functions by binding to its operator (TetO). The same principle can be applied to eucariotic promoters. In this case, two TetOs are located between the TATA box and site of initiation of transcription (+1) of the CMV promoter to create CMVtetO. In the absence of TetR (green ellipses) the CMVtetO promoter is fully functional, resulting in transcription and polyadenylation (pA) of the transgene (ORF). However in the presence of TetR, the TetOs are occupied and expression is off. The specificity and level of TetR repression can be determined by the addition of Doxycyclin (Dox). Dox (red rectangle) functionally inactivates TetR, thereby allowing the promoter to be fully active. The difference in expression levels +/- Dox represents the level of repression.

E1E4 Complementing Cell Line for Multiple Ad Serotypes

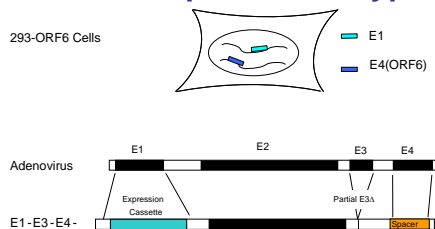


Figure 2. 293-ORF6 Cells Complement Multiple Ad Serotypes. 293-ORF6 cells have been used to manufacture Ad5 E1-E3-E4- vectors for multiple investigational drugs and vaccines now in clinical trials and are also compatible for the manufacture of Ad35 vectors. These cells are derived from HEK293 cells which express Ad5 E1 (E1a and E1b) and engineered to express Ad5 E4(ORF6). 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 (Poster 891) as well as Ad7 serotypes (Nan et al., Gene Ther. 2003 Feb; 10(4)). The use of 293-ORF6 cells as the base to construct a TetR cell line will allow the development of multiple Ad serotypes to express genes inhibitory to vector construction.

Constructing M2A TetR Cell Line

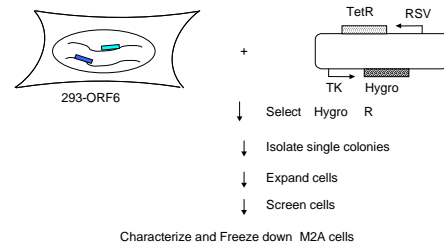


Figure 3. Constructing M2A cell line. M2A cell line was constructed by transfecting 293-ORF6 cells with a linearized plasmid that co-expresses TetR and a gene that confers Hygromycin resistance (Hygro R). After transfection the cells were placed under Hygro selection to obtain colonies arising from single cells. Individual colonies were picked, expanded and screened. A positive clone was then grown in the absence of drug selection before being further characterized for its ability to repress transcription from the CMVtetO promoter, maintain the TetR gene and support robust vector production.

Screening for M2A

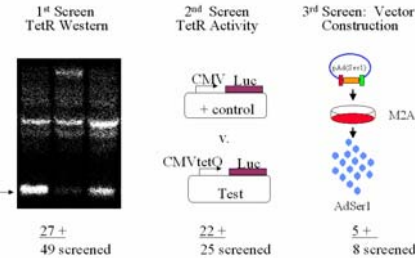


Figure 4. Screening for M2A. Three sequential screens were used to identify M2A. The 1st screen identified 27 positive of 49 screened clones to express TetR by Western Blot analysis. The arrow to the left of the Western Blot identifies TetR. (2nd screen) Of these 27 clones 25 were screened for their ability to repress luciferase (Luc) activity from the CMV promoter containing TetO (CMVtetO). This was accomplished by comparing Luc expression levels of a plasmid with the CMV (+ control) and CMVtetO (Test) promoter. A LacZ transfection efficiency control plasmid was also included. If levels of TetR were sufficient then expression from the Test plasmid would be reduced compared to the control plasmid. Of the 25 clones screened 22 were positive for repression. (3rd screen) Eight (8) of these 22 clones were tested for their ability to support generation of a vector with an inhibitory gene (AdSer1) that can not be constructed on 293-ORF6 cells. A linearized plasmid containing the entire AdSer1 genome (pAdSer1) was the starting material for this screen. Of the 8 clones, 5 supported the generation of AdSer1. One of these clones (M2A) was removed from Hygromycin selection and characterized for its ability to repress transcription during a viral infection, maintain the TetR gene and support vector production.

M2A Repress Transcription

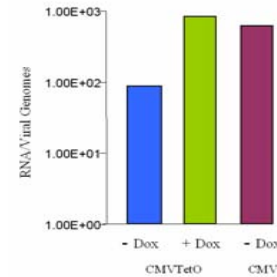


Figure 5. M2A Repress Transcription. Levels of repression were approximately 10 fold as determined using two different methods. The first method compared transcription levels from the CMVtetO promoter +/- Dox (green and blue respectively). The second was to compare the activity of the CMV (Burgandy) and CMVtetO (Blue) promoters. The experiment was carried out with two isogenic GFP expressing Ad5 E1-E3-E4- vectors that only differ in their promoter (CMV v. CMVtetO). M2A cells were infected with a multiplicity of infection of 1, harvested 12 hours later and levels of vector genomes and GFP RNA determined by real time quantitative PCR. Primers and probe to the pIX gene were used to quantitate vector genome levels while primer probes specific to GFP and a splice junction in the expression cassette (see figure 1) were used to quantitate RNA levels after a reverse transcriptase reaction. The levels of GFP transcripts were normalized to vector genome levels (RNA/Viral Genomes) and the results are shown above. Both methods of determining levels of repression (+/- Dox and CMVtetO/CMV) gave the same ~ 10X difference in expression levels. These data demonstrate the repression of the CMVtetO promoter in M2A cells is entirely due to TetR.

M2A Represses Transcription During Early and Late Infection

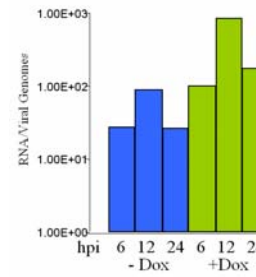


Figure 6. M2A Repress Transcription During Early and Late Infection. It is usually not known when during the viral life cycle that expression of an inhibitory gene has its deleterious effect on vector production. Using the same quantitative means and vectors as in figure 5, repression was found to occur 6, 12 and 24 hours post infection (hpi) [compare repressed (-Dox in blue) and not (+Dox in green)]. In fact, the level of repression is nearly the same early (6 hpi) as late (24 hpi) in infection. This is particularly surprising since there are 100X more genomes at 24 hpi than at 6 hpi (data not shown). If the only difference between early and late infection was viral copy number, then no repression should be detected late (5-10 fold repression at 6 hpi/100 times increase in genomes). The reason for the repression of transcription late in infection is under investigation. These data indicate that M2A cells can work against inhibitory genes that act early or late in the viral infection, thereby increasing the number of vectors available for investigation.

TetR Gene is Maintained in M2A

Passage	Copies TetR/C.E.
19	4.2 +/- 0.4
28	6.2 +/- 1.1
46	4.8 +/- 0.2

Figure 7. The TetR Gene is Maintained in M2A cells. To ensure the TetR gene is genetically stable, relative copy numbers were measured at different passages of M2A. The TetR copy number is calculated per Cell Equivalent (C.E) units using β -Actin as a reference standard. The TetR copy number is maintained for at least 25 passages (19-46) demonstrating M2A cells to be genetically stable.

Good Vector Productivity on M2A

AdSEAP	Yield pu/cell	AdSer1	Yield pu/cell
1	12.8 x 10 ⁴	1	3.4 x 10 ⁴
2	7.04 x 10 ⁴	2	7.6 x 10 ⁴
3	10.8 x 10 ⁴	Average	5.5 x 10 ⁴
Average	10.2 x 10 ⁴		

Figure 8. Good Vector Productivity on M2As. The ability to manufacture large quantities of vector is required for commercial purposes, particularly for vaccine products. The productivity of M2A was tested with two vectors, one with an inhibitory (Ser1) and non-inhibitory gene (SEAP). The average of three different experiments of AdSEAP on M2A cells generated 10.2 x 10⁴ particle units (pu)/cell. A similar average yield of 5.5 x 10⁴ pu/cell was obtained with AdSer1. The pu number was determined from crude lysate by HPLC using an Ad5 standard curve. Since large quantities of vector with inhibitory and non-inhibitory genes can be made this indicates that M2A cells could be used for manufacturing purposes.

Summary

- A number of transgenes are inhibitory to the construction of adenovirus vectors. The M2A cell line, which expresses TetR, overcomes this impediment.
- Properties of the M2A cell line.
 - Can construct E1-E3-E4- vectors with inhibitory genes expressed from the CMV promoter that were previously not possible to make.
 - Expresses TetR
 - Represses transcription from the strong CMV promoter.
 - Represses transcription during both early and late phase of infection.
 - Gives high vector yields (1x10⁵ pu/cell)
 - Complements E1-E4- vectors from multiple serotypes.
 - Made from the FDA approved 293-ORF6 manufacturing cell line for E1-E3-E4- vectors.
- The M2A cell line increases the therapeutic potential of Advectors by expanding their application to genes inhibitory to vector generation.

