

Development of Novel E1-Complementary Cells for Adenoviral Production Free of Replication-Competent Adenovirus

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Human adenoviruses have been used as highly efficient gene delivery vehicles or oncolytic agents in a variety of clinical and preclinical studies. Recombinant adenoviruses have predominantly been rendered replication defective or conditionally replicating via modifications to the E1 region, such as deletions, mutations, or replacement of the endogenous transcriptional control elements [1]. However, the E1 gene products are important for directing cellular and viral gene expression to enable a productive virus life cycle. During viral infection, E1A expression pushes the cell cycle into S phase to support viral DNA replication and E1B expression delays cellular apoptosis, thus gaining time for successful viral production [2]. Therefore, due to the essential role of E1 genes in adenoviral biology, the helper-independent production of E1-modified adenoviral vectors is assisted by cell lines that produce complementary E1 proteins.

Expression of E1A in transfected cells can immortalize rodent and human cells [3,4]. However, expression of E1A promotes apoptosis, which is inhibited by coexpression of E1B [5,6]. Therefore, cotransfection of E1A and E1B is necessary to establish stable cells that express both E1A and E1B *in trans*. The human embryonic kidney 293 cell line was generated about 30 years ago and since then it has been used as the major host cell for adenoviral production [7]. In 293 cells, a continuous adenovirus type 5 (Ad5) DNA fragment containing both E1A and E1B genes (nucleotides 1 to 4344) was integrated into chromosome 19 at 19q13.2 [8]. Since many first-generation adenoviral vectors are deleted from nucleotide (nt) 400 to nt 3500, the viral genome may have significant homology to the integrated viral DNA fragment in the host cell DNA. This homology between cellular DNA and viral genome can mediate double crossover recombination [9]. Therefore, 293-cell-derived viral E1 DNA may rescue the E1 deletion in the viral products and generate replication-competent adenovirus (RCA). This is problematic for large-scale viral production and clinical applications [9–11]. The fundamental concern for the presence of RCA in viral products is safety. The FDA has

established that the presence of RCA in replication-defective adenoviral vectors must be lower than 1 RCA in 3×10^{10} viral particles (vp) [12].

To reduce the generation of RCA during adenoviral production, other E1-complementary cells that contain a smaller adenoviral E1 DNA fragment to minimize the homology to the viral genome have been developed [13–17]. The reduction of the sequence common between cell and vector may eliminate homology at least at the 5' end of the adenoviral vector to reduce significantly recombination mediated by double crossover. For example, the Per.C6 cell line was generated using adenoviral E1 DNA that deleted the 5' endogenous E1A promoter and DNA 3' of the E1B 55-kDa coding region [14]. This cell line has limited homology to many E1-deficient adenoviral vectors and therefore significantly reduces RCA during vector production. However, the continuous E1 DNA fragment in the host cell DNA cannot preclude unconventional recombination. Only a short stretch of homology between cellular DNA and the vector genome can mediate recombination and generate recombinants with a rescued E1 region [9]. Although these recombinants are helper-dependent for a productive viral replication, their presence is still problematic for product qualification and clinical application.

The emergence of undesirable recombinants during viral production is also an important consideration for oncolytic viruses. These conditionally replication-competent adenoviruses are developed by viral gene mutation or replacement of transcriptional control elements for essential viral genes. Most of the modifications are in the E1 region and allow the viruses to replicate and kill specifically permissive tumor cells [1]. Although different permissive tumor cells can be used for production of these replicating viruses, a common production cell line may greatly reduce costs by standardizing process development and qualification. Based on the design of oncolytic adenoviruses such as CG7870, CV890, and CG8840 [18–20], E1-complementary producer cells can be used for the production of these viruses despite their

specificity for different cancers. However, the use of conventional E1-complementary cells like 293 cells, which have the continuous stretch of wild-type E1A and E1B genes, may generate wild-type RCA. This continuous DNA fragment may also generate other undesirable recombinants that alter the transcriptional control elements for E1A or E1B genes. The presence of these recombinants has a negative impact on viral tropism and potency, as well as product safety, and needs to be eliminated.

In this study, we developed novel E1-complementary cells for the production of RCA-free adenoviruses. We used the highly efficient retroviral transduction system *rkat43.2* [21] to generate retroviral vectors for adenoviral E1 expression. First, we constructed plasmids *rkat43.2E1A* and *rkat43.2E1B* (Fig. 1A) by replacing the CD4 ζ coding sequences of pRT43.2F3 [21] with the DNA coding for Ad5 E1A and E1B, respectively. We generated high-titer retroviral supernatants by transient cotransfection of *rkat43.2E1A* or *rkat43.2E1B* with MMLV packaging plasmids as previously described [22]. We then utilized the retroviral supernatants MMLV-E1A and MMLV-E1B to co-infect the human lung carcinoma cell line A549 by spinoculation. We used MMLV-E1A/MMLV-E1B ratios of 1/3, 1/1, and 3/1 to optimize the infection conditions for generating stable cells.

Under current study conditions, most of the cells in all three infections died during the first week. We single-cell cloned the remaining cells by limiting dilution and screened them with an E1-deleted, GFP-expressing adenovirus (Ad-GFP), which is capable of replication only in E1-producing cells. We harvested the crude viral lysates and used them to infect HuH7 cells, which we then assayed by FACS for GFP expression (Supplemental Fig. 1). In naïve A549 cells, Ad-GFP did not replicate and the subsequent HuH7 infection revealed only a background level of GFP-positive cells. In contrast, Ad-GFP actively replicated in 293 cells and therefore the subsequent HuH7 infection produced a gene transfer efficiency of 100%. Clones generated from co-infection of E1A/E1B retroviral vectors at a ratio of 1/3 and 1/1 supported Ad-GFP replication, whereas clones transduced with more MMLV-E1A than MMLV-E1B (3/1 ratio) could not. We selected the two clones from the 1/1 population with an efficient level of GFP expression, clones 51 (Ac51) at 91% and 139 (Ac139) at 78%, for further characterization. To ensure that the new clones were free of any replication-competent MMLV sequences, we isolated genomic DNA from naïve A549, Ac51, and Ac139 cells and analyzed it by TaqMan PCR with primers specific for MMLV gag/pol. Both parental A549 cells and adenoviral producer cell clones were negative for MMLV gag/pol sequences (data not shown).

To evaluate viral production quantitatively, we tested the new E1-complementary cells, Ac51 and Ac139, and 293 and Per.C6 cells for production of an E1-deficient

adenovirus encoding GM-CSF (Ad-GMCSF) (Supplemental Fig. 2A). Compared to the initial infection dose, both Ac51 and Ac139 had over 1000-fold amplification of Ad-GMCSF and produced virus at levels similar to those of 293 and Per.C6 cells (Supplemental Fig. 2B). We maintained the cell lines in continuous culture and reassayed them at weeks 5 and 10 to evaluate the stability of their E1 complementation. Yields of Ad-GMCSF virus were stable for Ac51 and Ac139 cells through 10 weeks of culture and remained comparable to those of 293 and Per.C6 cells (Supplemental Fig. 2B).

In contrast to the E1-deficient adenoviruses, the conditionally replicating oncolytic adenoviruses retain some or all of the E1 genes. We evaluated production of two oncolytic adenoviruses, CG8840 and OV945 (Supplemental Fig. 3A), on naïve A549, 293, Ac51, and Ac139 cells and determined virus yield by plaque assay on 293 cells (Supplemental Figs. 3B and 3C). In these studies, the virus production from Ac51 and Ac139 was equivalent to or greater than that from 293 and A549 cells, suggesting that these clones can effectively complement production of E1-modified oncolytic adenoviruses. In further studies, we adapted Ac51 and Ac139 cells to serum-free medium and they supported the production of E1-deficient and oncolytic viruses at levels comparable to those of 293 cells in suspension (Supplemental Figs. 3A and 3B).

We analyzed the expression of Ad5 E1 proteins from the new A549-derived E1 cells by Western blot (Fig. 1B). In this study, we visualized E1A, E1B 19-kDa, and E1B 55-kDa proteins with appropriate antibodies and compared them among the Ac51, Ac139, 293, and Per.C6 cells. Both Ac51 and Ac139 cells produce amounts of E1A higher than those of Per.C6 and 293 cells, while E1B 19-kDa production in all four cell lines is similar. However, the E1B 55-kDa production in both Ac51 and Ac139 cells is significantly lower than that of Per.C6 and 293 cells. Based on the structure of the E1B-expressing retroviral vector, the coding regions for both E1B 19-kDa and 55-kDa proteins overlap and their transcription is driven by the retroviral LTR to form the mRNA for both proteins. Further translation from the second AUG for the E1B 55-kDa protein may not proceed efficiently and thus results in low 55-kDa expression in the new E1 cells. In 293 or Per.C6 cells, the E1A and E1B genes were initially transfected as a continuous DNA fragment from the viral genome. Their E1 gene expression may still follow the temporal control mechanism used in the adenovirus, and E1B 55-kDa protein may be expressed in a more efficient manner [23]. While both Ac51 and Ac139 have lower production of E1B 55-kDa protein than 293 and Per.C6 cells, there is no impact on their growth kinetics compared to parental A549 cells. The expressed E1B 55-kDa products are sufficient for these cells to support adenovirus production, suggesting that E1B 55-kDa protein in these cells may have a less significant role in

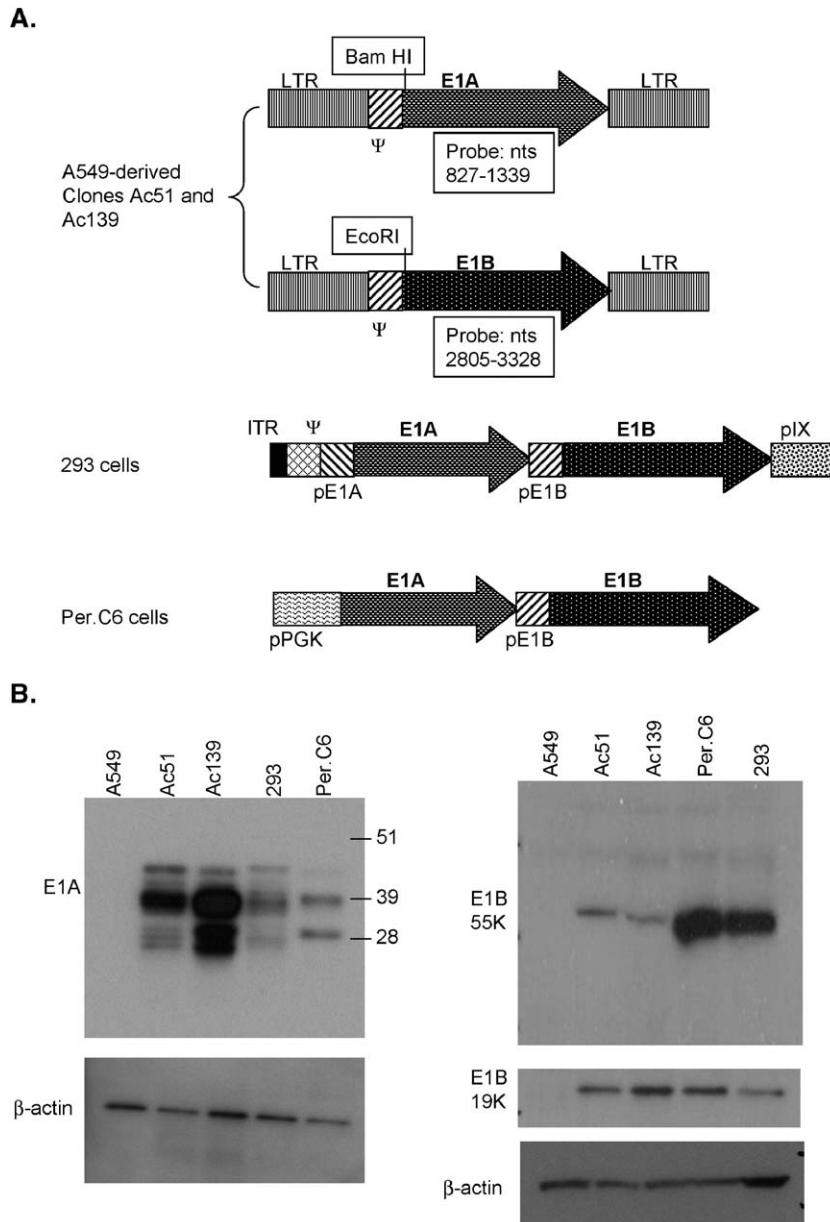


FIG. 1. E1A and E1B protein expression in clones Ac51 and Ac139 as assessed by Western blot. (A) Schematic structure of adenoviral E1 DNA in 293 cells, Per.C6 cells, and the new A549-derived clones Ac51 and Ac139. Clones Ac51 and Ac139 contain Ad5 E1A and Ad5 E1B introduced by simultaneous infection with separate MMLV E1A and E1B vectors. Two plasmids, *rkat43.2E1A* and *rkat43.2E1B*, were constructed by replacing the CD4 ζ coding sequences of pRT43.2F3 [21] with the adenoviral genome sequences coding for Ad5 E1A mRNA (Ad5 nucleotides 548–1575, GenBank Accession No.X02996) and Ad5 E1B open reading frame with part of pIX (nucleotides 1682–3825 of Ad5, GenBank Accession No.X02996), respectively. The infectious retroviral particles comprising the E1A and E1B expression vectors were produced by cotransfecting *rkat43.2E1A* or *rkat43.2E1B* plasmids with MCVecog/p and 6.1CMVamphoenv [22]. The resulting viral supernatants MMLV-E1A and MMLV-E1B were then utilized for cell transduction. 293 cells contain nucleotides 1 to 4344, from the 5' ITR to pIX. In Per.C6 cells the native E1 sequences upstream of the E1A gene have been replaced by the human phosphoglycerate kinase promoter (pPGK). The human embryonic kidney 293 cells and human A549 lung carcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (4.5 g/L glucose), 10% fetal bovine serum (JRH Biosciences, Inc., Lenexa, KS, USA), 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The human embryonic retinoblast Per.C6 cell line was obtained from Crucell (Leiden, Netherlands). These cells were cultured in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. (B) A549, Ac51, Ac139, 293, and Per.C6 cell lysates were prepared in lysis buffer supplemented with protease inhibitors. For E1A detection, 2 μ g of each lysate was separated on a 4–12% NuPage Novex Bis-Tris gel (Invitrogen, Carlsbad, CA, USA), transferred to an Invitrolon PVDF membrane (Invitrogen), and then probed with an E1A-specific antibody (Neomarkers, Lab Vision Corp., Fremont, CA, USA) and horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham, Piscataway, NJ, USA). For E1B detection, 25 μ g of each lysate was separated on a 4–12% NuPage Novex Bis-Tris gel (Invitrogen), transferred to an Invitrolon PVDF membrane (Invitrogen), and probed with antibodies specific for E1B 55K and E1B 19K proteins (Oncogene Research Products, Calbiochem, San Diego, CA, USA). Secondary probing was performed with horseradish peroxidase-conjugated antibodies: anti-mouse IgG (Amersham), anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-rat IgG (Boehringer Mannheim, Indianapolis, IN, USA). An enhanced chemiluminescence plus (ECL Plus; Amersham) method was used to detect the protein bands. Protein molecular weight markers (kDa) are labeled on the right side of the image. As a loading control, β -actin probing was completed on each of the blots described above using a β -actin primary antibody and anti-goat IgG horseradish peroxidase-conjugated secondary (Santa Cruz Biotechnology).

complementing viral production. Previously, similar low E1B mRNA, compared to the extent of E1A mRNA, was found in transfected E1-complementary cells [24]. In addition to the major species 289R, 243R, and 55R, other molecular weight bands, such as the minor species 217R and 171R, were visible by Western blot, especially when the gels were overloaded (see Supplemental Fig. 5).

The most significant improvement in these new E1-complementing cell lines is the genomic separation of the E1A and E1B genes achieved by the use of retroviral co-infection. To explore the relative positions of the E1A and E1B genes in the host cell genome, we analyzed the cellular DNA from Ac51, Ac139, 293, and Per.C6 by PCR and Southern blot. First, we PCR-amplified DNA from these cell lines and an A549 control using primers specific for either E1A or E1B gene. As shown in Fig. 2A, the A549 cells were negative for either E1A or E1B sequences, whereas the four E1-producing cell lines were positive for both E1A and E1B genes as expected. However, when we selected the primers from both E1A and E1B genes to amplify the continuous E1A to E1B fragment, Ac51 and Ac139 have a pattern different from that of 293 and Per.C6 cells. As seen on the right of Fig. 2A, primers 1460.138.3 (E1A, nt 1338) and 1460.138.6 (E1B, nt 2815) amplified a 1.5-kb DNA fragment from 293 and Per.C6 cells, but not the new E1 cells, indicating that Ac51 and Ac139 cells contain genomically separated adenoviral E1A and E1B genes.

In a Southern blot assay, we digested DNA from different passages of Ac51, Ac139, and 293 cells with *Bam*HI (E1A) or *Eco*RI (E1B), separated it on a 0.7%

agarose gel, and screened it with probes specific for E1A or E1B genes (Fig. 2B). Ac139 appears to have two copies of E1A and two copies of E1B. Ac51 has four copies (upper band is a doublet) of E1A and two copies of E1B. The blots also indicate that patterns for both Ac51 and Ac139 remain constant at early and late passage, again confirming that these clones are genetically stable.

An important focus in this study was to evaluate the production of RCA in the different cell lines. We used an RCA-free viral stock of an E1-deficient virus, Ad-GMCSF,

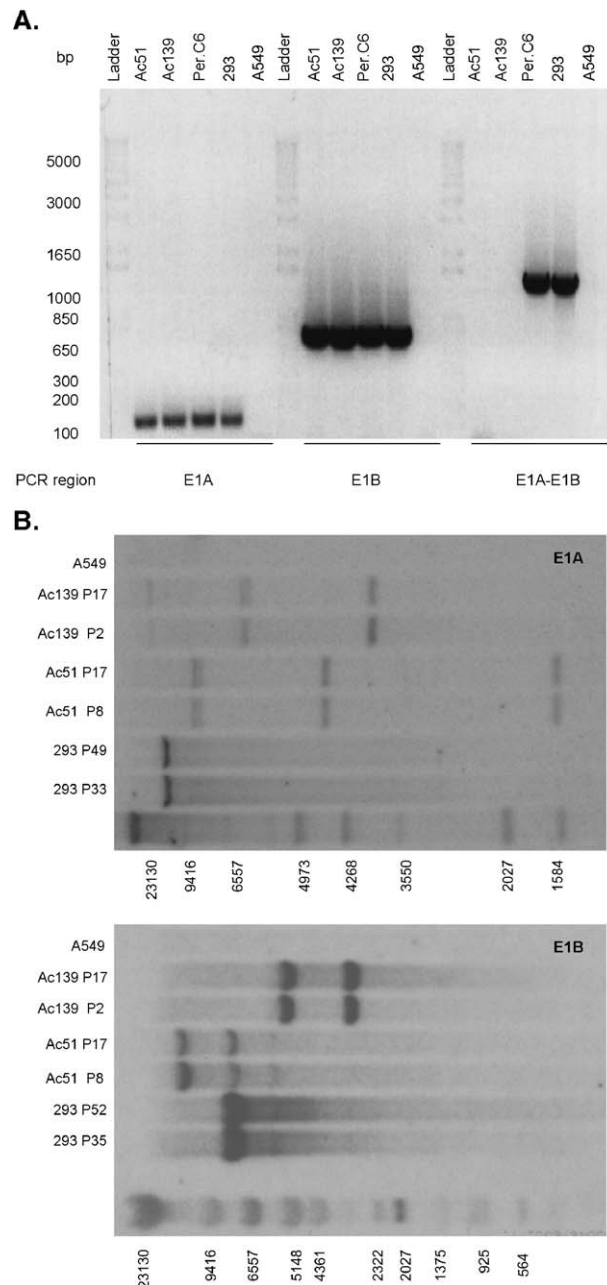


FIG. 2. Genomic analysis of E1A and E1B coding regions in clones Ac51 and Ac139. (A) DNA from A549, Ac51, Ac139, Per.C6, and 293 cells was analyzed by primers designed to detect E1A and E1B genes separately or E1A/E1B linked within the genome. DNA was purified from the cells with the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Primers 1460.138.3 (5'-TGTGTCTA-GAGAATGCAATAG) and 1460.138.4 (5'-TGGCCTGGGCGTTTACAGC) amplify the Ad5 E1A coding region (nt 1338 to 1542); primers 1460.138.5 (5'-ATGGAGCGAAGAAACCATCTG) and 1460.138.6 (5'-CCATAGAAGCTTACACCGTGTAG) amplify the Ad5 E1B coding region (nt 2019 to 2815), and primers 1460.138.3 and 1460.138.6 amplify Ad5 E1A (nt 1338) to E1B (nt 2815). For each PCR, cellular DNA was used in an Expand Long Template PCR system (Roche Diagnostics, Indianapolis, IN, USA) for 35 cycles. (B) DNA from early and late passages of A549, Ac51, Ac139, and 293 cells was analyzed by Southern blot using probes specific for E1A and E1B genes (Fig. 1A). Genomic DNA was extracted from the cells with a DNeasy Tissue Kit (Qiagen), digested with *Bam*HI (for E1A probing) or *Eco*RI (for E1B probing), separated on 0.7% TBE agarose gels, transferred to Nytran membranes (Schleicher & Schuell, Keene, NH, USA), and fixed by UV crosslinking. Adenoviral DNA from nt 827 to nt 1339 (GenBank Accession No.X02996) was prepared by double digestion of plasmid pXC1 (Microbix, Inc.) with *Bsp*EI/*Xba*I and used as a template for the E1A probe. Likewise, adenoviral DNA from nt 2805 to nt 3328 was digested from pXC1 by *Hind*III/*Bgl*II and used as the template for the E1B probe. Each template was isolated by gel electrophoresis and purified with a Gel Extraction Kit (Qiagen) and labeled with [³²P]dCTP using a Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA, USA). Following hybridization, membranes were exposed to a phosphor screen for autoradiography, and signals were detected on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

which met the FDA guideline (<1 RCA in 3×10^{10} viral particles) in a biological assay, for the RCA studies in Ac51, Ac139, 293, and Per.C6 cells. Ad-GMCSF has significant homology to 293 cells, as well as a 180-nt continuous DNA fragment homologous to Per.C6 cells. Therefore, we expected that when using 293 cells, RCA could be generated at a rate of 1 RCA in 1×10^5 to 1×10^{10} vector particles [25]. In Per.C6 cells, we also expected unconventional recombinants due to the single short stretch of homology [9]. In contrast, the genomic separation of E1A and E1B genes in the cellular DNA of clones Ac51 and Ac139 should greatly reduce the potential generation of E1-containing RCA or recombinants that may cause undesirable cytopathic effect in a biological assay.

To evaluate RCA status during viral production, we used the E1-deficient Ad-GMCSF virus to infect the four E1-complementing cell lines in a bioamplification study. The continuous amplification of first-generation vectors on different cells has previously been used for RCA evaluation [10,16]. In this continuous passage process, RCA or other forms of recombinants that emerge will be passed along with Ad-GMCSF to subsequent passages. Any recombinants with better replication efficiency than the parental Ad-GMCSF may be amplified during the passing and therefore can be detected with relative ease in the later passages.

We produced Ad-GMCSF for 20 passages in Ac51, Ac139, 293, and Per.C6 cells. To initiate passage 1 of the amplification, we infected each cell line with 2×10^8 vp of Ad-GMCSF at an m.o.i. of 75. We prepared crude viral lysates 72 h postinfection and determined titers by HPLC [26]. We then used these crude lysates for another set of identical infections on naïve cells. This amplification was repeated a total of 20 times. The viral production in each amplification cycle was $1\text{--}5 \times 10^{11}$ vp for all cell clones examined.

We performed PCR analysis of the viral crude lysates from 293, Ac51, and Ac139 cells at passages 0, 1, 9, and 18 to detect the emergence of RCA during the course of the amplification (Fig. 3A). The primer pair (66.114.2 and 49.17.2) used in this PCR assay specifically detects a 293-cell-derived E1 DNA fragment that overlaps the native E1A promoter and part of the E1A gene. The results show the expected positive 0.6-kb PCR band from the 293 cellular DNA, and there is no positive signal detected in the starting Ad-GMCSF (p0) sample. After multiple passages, positive PCR signals can be detected in samples derived from 293 cells at passages 9 and 18, but not in Ac51 or Ac139 cells, indicating that RCA has emerged during the viral amplification process. The sensitivity of this assay was 1 copy of wild-type Ad5 DNA in a background of 10^8 Ad-GMCSF viral DNA, and 10^9 copies of viral DNA (40 ng) were used in each PCR detection. Therefore, we estimated that there were at least 300 RCA in 3×10^{10} vp by passage 9 in samples derived from 293

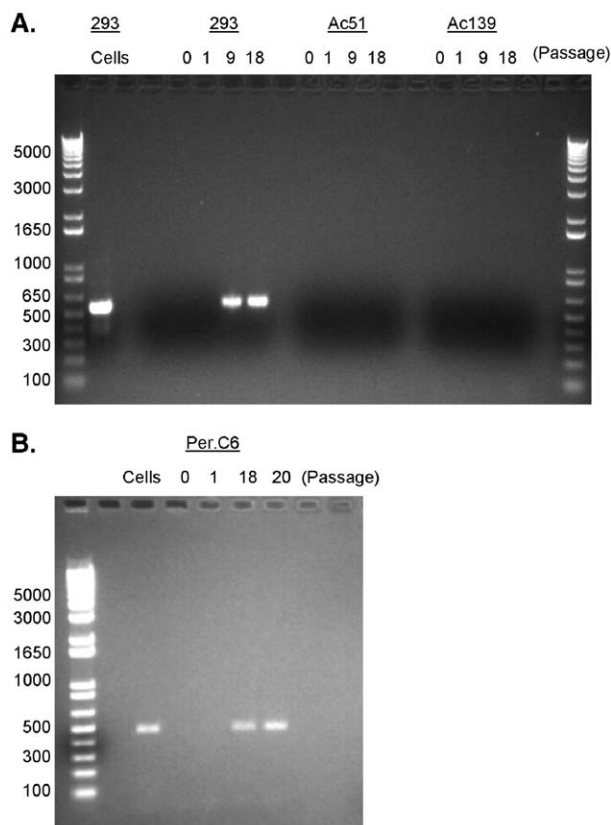


FIG. 3. PCR evaluation of RCA in bioamplified viral products. (A) Ad-GMCSF was serially amplified 20 times on 293, Ac51, and Ac139 cells. 2×10^8 vp of the Ad-GMCSF virus (<1 RCA in 3×10^{10} viral particles by a biological assay) was designated as passage 0 (p0) and used to infect 293, Ac51, and Ac139 cells at an m.o.i. of 75 vp for 72 h. The crude viral lysates were harvested and quantified by HPLC [26]. These p1 viruses were further applied to naïve cells of the same type under the same conditions for the next passage viral products. This process was repeated for a total of 20 passages. Crude viral lysates from passages 0, 1, 9, and 18 were pretreated with benzonase (Novagen, San Diego, CA, USA), and the viral DNA was extracted with Qiagen's Blood Kit (Qiagen). For each PCR detection, 40 ng of viral DNA sample was used as template. Primer pair 66.114.2 (Ad5 nt 133 to 153, 5'-GTGGCGAACACATGTAAGCG) and 49.17.2 (Ad5 nt 696 to 674, 5'-AGTTCGTGAAGGGTAGGTGGTTC) were selected for RCA detection from 293-cell-derived viral passages. (B) Ad-GMCSF was serially amplified 20 times on Per.C6 cells as described above, and crude viral lysates from passages 0, 1, 18, and 20 were examined by PCR using a primer pair specific for Per.C6-cell-specific E1 DNA. To detect recombinants that emerged specifically from Per.C6 cells, a previously published primer pair (PGK promoter, 5'-GGCTCCCTCGTCCGAAT, and E1A gene, 5'-CGGTACAAGTTTGGCATAGA) was employed [27].

cells. No positive signals were detected in any passage of Ad-GMCSF viral products derived from the Ac51 or Ac139 cells.

We conducted a PCR assay using a previously published primer pair specific for the phosphoglycerate kinase (PGK) promoter and E1A gene to examine the viral crude lysates from Per.C6 cells for recombination events [27] (Fig. 3B). In this study, viral DNA samples from passages 0, 1, 18, and 20 were prepared as described

TABLE 1: Biological RCA detection by hexon staining after bioamplification of E1-deficient virus on the different cell lines

Sample ^a	Hexon-positive plaques ^b
Naïve A549 cells only	0
Ac51	0
Ac139	0
293	5,440
Per.C6	32
Ad-GMCSF + 2000 Ad5	>20,000
Ad-GMCSF + 200 Ad5	>20,000
Ad-GMCSF + 20 Ad5	20,000
Ad-GMCSF only	0

^a RCA-free Ad-GMCSF virus (<1 RCA in 3×10^{10} viral particles) was used to infect 293, Per.C6, Ac51, and Ac139 cells in 6-cm plates at m.o.i. 75 vp for 72 h. Crude viral lysates were harvested and quantified by HPLC [26]. These p1 viruses were further applied to naïve cells of the same type under the same conditions for the next-passage viral products. This process was repeated for a total of 20 passages. For biological detection of potential RCA, 2×10^{10} vp of each p20 viral sample were amplified on 6×10^7 A549 cells plated in three T300 flasks 1 day prior to infection. The infection was performed in 20 ml DMEM with 2% FBS, and the flasks were incubated on a low-speed shaker for 48 h and then supplemented with an additional 30 ml of complete medium with 10% FBS for a total of 8 days. Cells were harvested in 10 ml for each p20 viral sample and crude lysates prepared. Controls were set up in parallel by spiking 2000, 200, 20, or 0 wild-type Ad5 into 2×10^{10} vp of p0 Ad-GMCSF virus and amplified through A549 cells in the same pattern.

^b For the viral plaque assay, 6-cm plates of A549 cells were infected with 1 ml of each viral crude lysate in triplicate. After 48 h incubation, the infection media were removed and cells were overlaid with medium containing 0.7% low-melting-point agarose (Invitrogen) for plaque formation. The agarose overlay was removed after 8 days and cells were fixed with 100% methanol. All the plates were stained for hexon with an Adeno X Rapid Titer Kit (BD Bioscience, San Jose, CA, USA) to visualize the viral plaques for quantification.

above. The positive PCR signal, a 0.5-kb DNA band, was detected in the Per.C6 cellular DNA and in viral passages 18 and 20. Sequencing of the PCR band confirmed that E1A and the human PGK promoter DNA was present in Ad-GMCSF viral products, indicating the presence of recombinants. Based on previous studies, the recombinants most likely contain E1 sequences derived from Per.C6-cell-derived DNA [9,27].

Although PCR evaluation has suggested that Ac51 and Ac139 do not produce RCA during viral production, we conducted a further biological assay to assess comprehensively the RCA status of these cells. We amplified further 2×10^{10} virus particles from each of the Ad-GMCSF p20 samples generated above on 6×10^7 naïve A549 cells for 8 days. We prepared crude lysates and assayed them on naïve A549 cells for plaque formation and hexon staining. To establish the sensitivity of the assay, we spiked 2×10^{10} Ad-GMCSF particles with wild-type adenovirus and ran them in parallel. As shown in Table 1, for both 293 and Per.C6 cells, clear plaque-forming recombinants are present in the Ad-GMCSF products, indicating the presence of RCA or undefined recombinants. However, in an assay that was able to detect 20 wild-type particles in a background of 2×10^{10} Ad-GMCSF particles, we detected no viral plaques in virus amplified on Ac51 or Ac139 cells, suggesting that both cell lines allow RCA-free adenoviral production.

Taking the PCR and biological assay results together, there is strong evidence that Ac51 and Ac139 cells do not produce RCA during the production of E1-deficient adenoviral vectors. We also conducted a PCR evaluation of the oncolytic viral products produced from Ac51 and Ac139 cells. When we used retroviral vector-specific and E1B-specific primers in a PCR aimed to detect cellular-derived DNA fragments in OV945 product, no positive signal could be detected, suggesting that no cellular DNA-derived recombinant was present in the preparation of OV945 (data not shown).

In conclusion, we have successfully applied a novel strategy and used retroviral vectors to establish new E1-complementary cells. These cells have adenoviral E1A and E1B genes integrated into separate genomic locations. They are E1 complementary and support the production of E1-defective and E1-modified adenoviral production. Comprehensive RCA evaluation demonstrated that the new E1 cells do not support RCA generation. Further, adaptation of these E1-complementary cells to serum-free suspension culture makes them suitable for large-scale adenoviral manufacturing.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2006.02.020.

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