ABSTRACT

pAdvantage is a rapid cloning system for generating recombinant adenoviruses. The system is based on manipulating the full-length adenovirus genome as a stable plasmid in E. coli using intron-encoded endonucleases. These intron-encoded endonucleases cut their recognition sequences, which range from 15–39 bp, with high specificity. Their unusual long homing sequence makes them rare-cutting and ideal to use as cloning sites. We report how transgenes can easily be cloned directly into the E1 region of an adenoviral plasmid, followed by transfection into a mammalian packaging cell line, to produce homogeneous recombinant viruses without the need for plaque purification.

INTRODUCTION

Adenoviruses are double-stranded linear DNA viruses with a 36-kb genome. Several features of adenovirus have made them a popular choice as a transgene delivery vehicle for therapeutic applications. They have a broad host range, can easily be propagated and purified (4) and have a reasonable cloning capacity. Up to 7.5 kb of heterologous sequence can substitute for the E3 and E1 regions of adenovirus using complementation in 293 cells (5). Deletions in E4 together with a complementing E4/E1 cell line permits the inclusion of larger inserts up to 10 kb (6).

The most common approach in generating adenovirus recombinants involves in vivo homologous, intramolecular recombination between overlapping Ad sequences in plasmids and restriction-cleaved Ad viral DNA (10). However, problems with this approach include high background due to contaminating parental virus, which necessitates repeated rounds of plaque purification, and the low efficiency of in vivo homologous recombination. Other recombinant adenoviral systems have been developed to overcome some of these technical challenges. Several current methods involve generating recombinant adenoviruses utilizing the rec system in Escherichia coli. (1,6).

The method described in this report utilizes the homing sequence of the intron-encoded endonuclease I-Ceu I as a cloning site for direct transgene insertion into a viral plasmid.

Intron-encoded endonuclease is enzymes that are encoded within a group I intron, which mediates the intron’s transposition (9). The intron-encoded endonucleases have an unusual long homing sequence ranging from 15–39 bp, which makes them rare-cutting and ideal to use as cloning sites. The intron encoding I-Ceu I is called CeLSU.5 and is found in the large subunit rRNA gene of Chlamydomonas eugametos (7,8,11). The encoded protein I-Ceu I has a 26-bp homing site 5’-TAACTATAACGGTCCTAAGGTAGCGA-3’ in which we report to be a very good cloning site for the generation of recombinant adenoviruses.

MATERIALS AND METHODS

Plasmid Constructions

The shuttle plasmid pSV-ICEU I was constructed within a pBluescript SK(+) (Stratagene, La Jolla CA, USA) backbone. The cytomegalovirus (CMV) promoter, multiple cloning sites and the bovine growth hormone (BGH) poly(A) were polymerase chain reaction (PCR)-amplified from pcDNA3 (Invitrogen, Carlsbad, CA, USA) using the primers: 5’SV-, 5’-TGCACCGGT-AACCTGTAAGCTTAAATGATTGGC-3’ and 3’SV-, 5’-TGCACCGGT-AACCTGTAAGCTTAAATGATTGGC-3’. The method described in this report utilizes the homing sequence of the intron-encoded endonuclease I-Ceu I as a cloning site for direct transgene insertion into a viral plasmid.
cloned into BssHII-digested pBlue-script SK(+) to generate pSV-ICEU I. The PCR-amplified portion was sequenced on a Model 377 Sequencer (PE Biosystems, Foster City, CA, USA) to verify sequence integrity. The plasmid pSV2-ICEU I (Figure 1B) is derived from pSV-ICEU I and contains a mutation (TACGTA to TACGA) that eliminates the SnaBI site in the CMV promoter. This was accomplished by replacing a KpnI-NdeI fragment from pSV-ICEU I with a KpnI-NdeI fragment from the plasmid called pCMV (Gary White, Genzyme, unpublished), which carries the mutated SnaBI site within the CMV promoter. The viral vector pAdvantage was constructed in a multi-step manner, as depicted in Figure 1A. First, pAd2-ICEU I was generated from its parent vector pAd (D. Souza, unpublished). pAd is a pBr322-backbone-based vector, which contains Ad2 sequence nucleotides (nt) 1–357 and nt 3328–10685. A polylinker containing unique restriction sites supplants the Ad2 E1 region. An oligonucleotide containing the I-CeuI recognition site was cloned into the SpeI and MluI within pAd polylinker to generate pAd2-ICEU I.

Second, a SnaBI site was created just outside the inverted terminal repeat (ITR) in pAd2-ICEU I by PCR. The SnaBI recognition site was included within the 5′ PCR oligonucleotide that was used to amplify the ITR. The ITR PCR product was cloned into the unique EcoRI and SpeI sites of pAd-ICEU I, replacing the former ITR to create pAd2-ICEU I-SnaBI.

Third, the vector pAdE4/E3-2.9 (Cathy Sookdeo, Genzyme, unpublished), which contains Ad2 sequence from nt 23428 to 3′ ITR with a deletion in E3 from nt 27971–30937, was modified to create a SnaBI site flanking the 3′ ITR by PCR. The ITR was amplified with a 5′ oligonucleotide that introduced the SnaBI site. The ITR product was cloned into the unique SalI and AvrII sites of pAdE4/E3-2.9, replacing the existing ITR to create pAdE4/E3-2.9 SnaBI.

A triple ligation of BamHI-BsrBI cut pAd2-ICEU I- SnaBI vector with a 5.4-kb BamHI-SrfI fragment from pAdE4/E3-2.9 SnaBI and a 17-kb BsrBI-SrfI fragment from Ad2 was executed to generate pAdvantage. The ligation was transformed into DH5™ competent cells (Life Technologies, Gaithersburg, MD, USA). Putative recombinant clones were screened by standard mini-prep analysis.

[The pAdvantage vector, the shuttle vector and any precursor vectors will be made available to all interested readers after they fill out a standard material and transfer agreement.]

Preparation of pAdvantage for Cloning

Five micrograms of pAdvantage were digested with a total of 30 U (30 µL) I-CeuI (New England Biolabs) in 500 µL 1× I-CeuI buffer at 37°C over 3 h. Ten units of I-CeuI were added every hour to achieve a total of 30 U. Twenty units (1 µL) alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany) were then added, and the reaction was incubated at 37°C for an additional 20 min. The DNA was extracted twice with equal volumes of (25:24:1, vol/vol) phenol:chloroform:isoamyl alcohol (Life Technologies) and then precipitated by adding 1/16 vol of 5 M NaCl and 2 vol of 100% ethanol. The DNA pellet was resuspended in 20 µL 1× TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Two microliters (0.25 µg) of vector were used for cloning with threefold molar excess of insert in a total volume of 50 µL of 1× T4 ligation buffer and 1 µL high-concentration T4 ligase (New England Biolabs). The reaction was incubated at room temperature for a minimum of 1 h.

Cell Culture Medium and Reagents

The 293 cell line was purchased from ATCC (Rockville, MD, USA) and was propagated in Dulbecco’s modified Eagle medium (DMEM; high glucose)
Figure 1. Overview of the pAdvantage System. (A) Adenoviral cloning vector pAdvantage. (B) Shuttle vector pSV2-ICEU1. (C) Recombinant adenoviral vector harboring the CFTR gene with the ΔF508 mutation.
with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies).

Viral Packaging in 293 Cells

Twenty micrograms of plasmid DNA were digested with 5 µL (20 U) SnaBI in 100 µL 1× NEB No. 4 Buffer (New England Biolabs) at 37°C for 1 h. The DNA was precipitated with 6 µL 5 M NaCl and 200 µL ethanol and collected by microcentrifugation at 14,000 rpm in a Model 5415C Centrifuge (Eppendorf, Madison, WI, USA) for 10 min. The 293 cells seeded in a 10-cm plate at 2 × 10⁶ cells per plate were transfected with SnaBI-digested DNA using the MBS Mammalian Transfection Kit (Stratagene). Plaques were picked 10 days post-transfection by 10-mm cloning cylinders in a total volume of 200 µL DMEM plus 10% FBS per plaque.

Isolation of Viral DNA

Six-well plates were seeded with the 293 cells at a density of 2.5 × 10⁵ cells per well. The cells were infected the next day with 100 µL putative recombinant viral plaque. When cytopathic effect (CPE) was visible (generally about 3–5 days post-infection), cells and supernatant were harvested. The supernatant was saved as a source of virus, while the cells were pelleted and resuspended in 650 µL Hirt lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.6% sodium dodecyl sulfate [SDS]). One hundred and sixty three microliters of 5 M NaCl were then added, and samples were incubated at -20°C for 10 min. Cellular debris was pelleted at room temperature by microcentrifugation at 14,000 rpm as above for 15 min. The lysate was transferred, leaving cellular debris behind, and was incubated at 37°C for 1 h with proteinase K (Life Technologies) at a final concentration of 200 µg/mL. Samples were extracted once with an equal volume of (25:24:1, vol/vol) phenol:chloroform:isoamyl alcohol, and the DNA was precipitated with the addition of 2 vol of 100% ethanol. DNA pellets were resuspended in 50 µL of 1× TE, and 5 µL were used for restriction analysis.

Note: For 10-cm plates, cells were seeded at a density of 1 × 10⁶ per plate, and the reagent amounts were doubled.
RESULTS AND DISCUSSION
Design of Cloning Vectors for Recombinant Adenovirus Construction

The adenoviral vector and shuttle vector were constructed in a stepwise manner (Figure 1, A and B) as described in Materials and Methods. This quick cloning system involves three steps. (i) The transgene is cloned into the shuttle vector pSV2-ICEU I. (ii) It is then subcloned from pSV2-ICEU I by I-Ceu I digestion into the I-Ceu I site of the viral vector pAd_vantage. (iii) The pAd_vantage-based recombinant adenoviral vector is cleaved with SnaBI to expose the inverted terminal repeats and is then transfected into 293 cells for generation of virus. Viral plaques are visible within seven to ten days. The total time to generate a recombinant virus is about three weeks.

Generation of Recombinant Adenovirus Ad2 CMV CFTR ΔΔF508 ΔΔE3-2.9

To test the efficiency of the system, a cystic fibrosis transmembrane conductance regulator (CFTR) ΔF508 recombinant adenovirus was generated. Since the CFTR gene is 4.5 kb in size, it was chosen as a practical virus to make, demonstrating the flexibility of the system to accommodate large inserts. A CFTR gene containing the ΔF508 mutation was cloned into the XbaI-ApaI sites of pSV2-ICEU I as a 4.5-kb SmaI-ApaI fragment from pCMVCFTR 936c ΔF508 (D. Souza, unpublished). The gene was then subcloned into pAd_vantage to generate pAd_vantage-CFTR 508 (Figure 1C). One out of sixteen putative pAd_vantage-CFTR 508 recombinant colonies screened were positive for transgene insertion, resulting in a 6% cloning efficiency. The clone appeared to be stable in E. coli, as no rearrangements were detected by restriction analysis (data not shown). The low efficiency for this cloning was a result of either a poorly dephosphorylated vector or uncleaved vector background. The method for preparing the viral vector has been improved since this construct was made. To date, based on five independent experiments, we achieve a 53% cloning efficiency. The method for preparing the viral vector stated in Materials and Methods is current.

DNA from 1 L of a pAd_vantage-CFTR 508 culture was isolated using the QIAfilter™ Plasmid Mega Kit and QIAGEN®-tip 2500 (Qiagen). Twenty micrograms of DNA were digested with SmaI and then transfected into 293 cells. At day seven, one plaque was picked to infect a single well from a 6-well plate. When most of the cells had rounded and detached from the plate (see Materials and Methods), cells were harvested for Hirt analysis.

Since the virus is cloned, it is not necessary to pick isolated plaques. The whole viral population should be homogeneous, and, therefore, the entire plate can be harvested. However, we
have found that we obtain confirmation of viral identity and virus expansion quicker if the first visible plaque is picked and used to infect a small population of cells (6-well seeded at 2.5 × 10^5 cells per well) rather than wait for the entire plate to develop CPE.

Structure of Ad2CMVCFTR ΔF508 ΔE3-2.9 was confirmed by restriction analysis of Hirt DNA (Figure 2). Lanes 2 and 3 represent parental virus Ad2ΔE1ΔE3-2.9 (pAd\textsubscript{vantage}) and Ad2CMVCFTR ΔF508 ΔE3-2.9 Hirt DNA digested with BamHI, respectively. As observed, fragments at 11.4, 7.8, 6.2 and 4.7 kb were detected for Ad2ΔE1ΔE3-2.9, and additional bands characteristic of CFTR at 2.5, 1.5 and 1.1 kb were detected for Ad2CMVCFTR ΔF508 ΔE3-2.9. Lanes 4 and 5 represent parental virus Ad2ΔE1ΔE3-2.9 and Ad2CMVCFTR ΔF508 ΔE3-2.9 Hirt DNA digested with PshAl, respectively. As observed, fragments at 27 and 3.0 kb were detected for Ad2ΔE1ΔE3-2.9, while fragments at 27 and 8.7 kb were detected for Ad2CMVCFTR ΔF508 ΔE3-2.9. Based on the BamHI and PshAI diagnostic digestions, the CFTR recombinant virus generated appears to contain the correct genomic structure and is free from parental virus contamination.

**Expression of Recombinant Adenovirus Ad2CMVCFTR ΔF508 ΔE3-2.9**

We next wanted to demonstrate transgene expression from Ad2CMVCFTR ΔF508ΔE3-2.9 generated with the pAd\textsubscript{vantage} system. For this purpose, 293 cells were infected with Ad2CMVCFTRΔF508ΔE3-2.9 or Ad2CFTR-16 at a multiplicity of infection (MOI) of 10. Sodium butyrate was added to the media at a final concentration of 5 mM to enhance transcription and thus facilitate detection of ΔF508 CFTR. Cells were harvested 72 h post-infection to determine CFTR production by in vitro phosphorylation assay as previously described (2). Lanes 3 and 4 in Figure 3 indicate the characteristic band C observed for the mature form of CFTR, while lanes 1 and 2 show only band B form, which is consistent with the ΔF508 mutation (3).

**Second Generation pAd\textsubscript{vantage} Vectors**

The system is limited to expression cassettes that do not contain a SnaBI. At the time the system was designed, we were concerned with the effect that DNA bases that flank the viral ITR’s had on viral DNA replication. Upon consulting the literature, we decided that the first generation viral vector should contain a restriction endonuclease recognition site that leaves the minimum number of bases that flank the viral ITR’s after enzyme excision of viral DNA from plasmid backbone to minimize possible inhibition of viral DNA replication. Subsequent viral vectors would address the tolerance of additional flanking DNA bases on viral DNA replication.

We are currently testing whether an 18-bp recognition site for the restriction encoded endonuclease I-PpoI would prove fruitful, thus allowing all vectors to be released. With the current pAd\textsubscript{vantage} Vector presented in this manuscript, most expression cassettes can be released. Site-directed mutagenesis can remedy gene cassettes that contain a SnaBI site. We are also working on a second-generation pAd\textsubscript{vantage} Vector that will allow for transgene insertion not only in the E1 region but in the E3 and E4 regions as well. We are screening other commercial intron-encoded endonucleases that will serve as effective enzymes for the manipulation of full-length adenovirus genome in E. coli.

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