Simultaneous Insertion of Two Expression Cassettes into Adenovirus Vectors

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ABSTRACT

*We have designed AdenoQuick, a fast and versatile method to construct first-generation adenoviral vectors that contain one or two transgenes in the E1 and/or the E3 region. The method is based on the reconstitution of the entire genome of the desired recombinant virus in *E. coli* and the subsequent transfection of the DNA in a helper cell line. Since the construction of large adenoviral plasmids is generally difficult and therefore rebuffering for inexperienced researchers, we have optimized the cloning strategy by using bacterial positive-selection markers and a set of specific restriction enzymes that allow for directional cloning. The system is 99% efficient and allows one to insert simultaneously two expression cassettes into the E1 and E3 regions of the adenovirus genome.*

INTRODUCTION

Adenoviral vectors have become useful tools to introduce genes into mammalian cells, primarily because they can infect a large variety of resting and dividing cells, and they are easy to grow to high titers (3). First-generation adenoviral vectors are deleted for the E1 and/or E3 regions and can accommodate DNA inserts up to about 8.3 kb (4). Several straightforward methods are now available to construct such vectors (1,2,8,13–15). The techniques that are based on the reconstitution of the genome of the desired recombinant virus in *E. coli* and the transfection of the resulting plasmid in helper cells (5–8,11,13,16) have several advantages, including (i) rapid virus rescue, (ii) the possibility to manipulate the adenovirus genome in regions other than E1, and (iii) in particular, an apparently easier recovery of recombinant viruses expressing toxic genes (17). However the construction of large plasmids in *E. coli* is labor intensive, difficult, and rebuffering for inexperienced researchers. Up to three cloning steps in *E. coli*, a colony screening, and a high-quality plasmid DNA preparation are generally required for every desired recombinant virus.

To facilitate and accelerate the construction of large adenoviral plasmids, we have designed a new set of vectors that contain interrupted palindromic restriction sites. By varying the nature of the three base pairs composing the cohesive end, we have created an efficient directional cloning system, which allows one to insert two transgenes into the adenoviral genome in the same ligation reaction.

MATERIALS AND METHODS

Plasmids

pE1.1, pE1.2, and pE3.1 are the shuttle plasmids in which the transgenes must be inserted before being transferred into the large adenoviral plasmids. pE1.1 (Figure 1) contains the first 353 nucleotides from the Ad5 genome, preceded by two rare-cutting enzymes (*PacI* and *SwaI*) and followed by a multiple cloning site. This plasmid contains, in addition, a *cos* site for DNA packaging into phage λ , a pUC19-derived origin of replication, and a kanamycin-resistance gene. pE1.2 (Figure 2) is identical to pE1.1, except for the deletion of the *cos* site. pE3.1 (Figure 2) is a pUC19-derived plasmid that contains a multiple cloning site flanked by a 180-bp λ *cos* site and a kanamycin-resistance gene. In all shuttle plasmids, the sequences encompassing the kanamycin-resistance gene, the λ *cos* site, the adenoviral sequences, and the multiple cloning site (when present) are flanked by two sets of restriction sites *AlwNI*, *BstAPI*, *DraIII*, and *PfIMI*. Within one set, the sticky ends generated by these enzymes are identical to each other but are incompatible with those generated in the other set (GAG vs. AGA in pE1.1 and pE1.2; CCA vs. ATG in pE3.1).

The adenoviral plasmids AdenoQuick1.1, AdenoQuick1.2 (Figure 1), and AdenoQuick13.1 (Figure 2) contain the right end of the Ad5 genome (starting at nucleotide 3504), an ampicillin-resistance gene, a pUC19 origin of replication, and sequences from phage λ as stuffer DNA. The right inverted terminal

Figure 1. Strategy for the construction of E1-substituted adenoviral vectors. In each vector, the ITRs are indicated by triangles and the multiple cloning sites are indicated by a black box. The L-shaped boxes indicate the cohesive ends generated after cleavage with *PflMI*, *DraIII*, *BstAPI*, *AlwNI*, or *SfiI*. Different tones indicate incompatibility. The other elements are self-explanatory. Refer to the text for the details of the strategy.

repeat (ITR) is flanked by *PacI* and *SwaI* sites. The two *SfiI* sites naturally present in wild-type Ad5 DNA were mutated by substituting A for G and C at positions 16291 and 16294 in the Ad5 genome, and C and G for, respectively, G and C at positions 23001 and 23004 in the Ad5 genome, introducing silent mutations in the adenovirus pVII and DNA-binding protein coding sequences. Two new *SfiI* sites were inserted in place of the E1 region. These sites generate sticky ends that are incompatible with each other but are complementary with those generated by digesting pE1.1 and pE1.2 with *AlwNI*, *BstAPI*, *DraIII*, or *PflMI*. AdenoQuick1.2 is identical to AdenoQuick1.1, except for a 2.7-kb deletion in the E3 region. AdenoQuick13.1 is a 37-kb plasmid similar to AdenoQuick1.2, except for the presence of two additional *SfiI* sites in the E3 region, which generate sticky ends that are incompatible with each other and with those present in the E1 region but are compatible with those generated by digesting plasmid pE3.1 with *AlwNI*, *BstAPI*, *DraIII*, or *PflMI*. The maximal transgene capacities of AdenoQuick1.1, AdenoQuick1.2, and AdenoQuick13.1 are about 5.2, 7.9, and 7.7 kb, respectively.

Cosmid Constructions and Purifications

Two to five hundred nanograms of DNA fragment(s) obtained by digesting the shuttle vectors with *AlwNI*, *BstAPI*, *DraIII*, or *PflMI* are ligated with the same amount of *SfiI*-digested AdenoQuick1.1 (intact E3 region), AdenoQuick1.2, or AdenoQuick13.1 (2.7-kb E3 deletion). The ligated DNAs are introduced into *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA) or DH5 α either by electroporation using an Electro Cell Manipulator[®] 600 (BTX, San Diego, CA, USA) or via packaging into phage λ using Gigapack[®] III packaging extract (Stratagene, La Jolla, CA, USA) and following the protocols provided by the manufacturers. Transformants are selected on LB-agar supplemented with 50 μ g/mL ampicillin and 25 μ g/mL kanamycin. Cosmid DNA is amplified in LB-Lennox supplemented with 25 μ g/mL kanamycin. Bacteria are harvested before the stationary growth phase is reached (i.e., when A_{600} =

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2.0–2.5). Good-quality cosmid DNA is obtained using anion-exchange columns such as BioMidi™ (Biosepra, Malborough, MA, USA), Nucleobond® (Clontech Laboratories, Palo Alto, CA, USA), or Plasmid Midi kit (Qiagen, Valencia, CA, USA), silica-based columns such as Wizard® PureFection (Promega, Madison, WI, USA), or double CsCl gradient centrifugation. Using Biosepra columns, 1 mL bacterial culture can yield up to 3 µg cosmid DNA.

Cell Culture and Transfections

Low-passage 293 cells are purchased from ATCC (CRL-1573) and cultured in DMEM (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS) (Sigma, St Louis, MO, USA), 4 mM glutamine, 1% penicillin-streptomycin (Life Technologies), and 4.5 mg glucose/L. Linearized cosmid DNA is transfected into 293 cells using the Ca₂P₄ method, as described previously (8).

Reporter Genes and Assays

Cassettes expressing firefly and *Renilla* luciferases were obtained, respectively, from plasmids pGL3 (2.2-kb *Clal* fragment) and pRLSV40 (1.8-kb *Bgl*III-*Bam*HI fragment) (Promega). Luciferase assays were performed according to the protocol provided by Promega.

RESULTS

Strategies

Like other methods, the AdenoQuick cloning system relies on the reconstitution of the entire genome of the recombinant virus in a plasmid and the rescue of the virus after transfection of the DNA into helper cells. Its originality lies in a two-step cloning procedure in *E. coli*, which greatly facilitates the isolation of the correct clone. This system is capable of efficiently and simultaneously introducing two expression cassettes into the adenovirus genome in a single ligation reaction.

In its simplest version, this system allows the construction of adenoviral vectors containing a single transgene in the E1 region (Figure 1). The first step

is the insertion of the expression cassette of interest into the multiple cloning site of the shuttle plasmid pE1.1, in place of the E1 region and close to two bacterial positive-selection markers: a kanamycin-resistance gene (Kan^r) and a λ *cos* site. These sequences are flanked by two sets of *Alw*NI, *Bst*API, *Dra*III, and *Pfl*MI sites. The recognition sites of these restriction enzymes are interrupted palindromes. All sites belonging to the same set generate nonpalindromic sticky ends that are identical to each other but are incompatible with those generated in the other set. The nonpalindromic nature of the cohesive ends will ensure the directional insertion of the expression cassettes into the adenoviral plasmids during the second cloning step and will prevent the formation of dimers or self-circularized products during the ligation reaction.

In the second step of the procedure, the complete genome of the recombinant adenovirus is reconstituted in a plasmid. The intermediate vector pE1.1 is digested with *Alw*NI, *Bst*API, *Dra*III, or *Pfl*MI (whichever is not present in the expression cassette), and the fragment containing the expression cassette is ligated with *Sfi*I-digested AdenoQuick1.1 or AdenoQuick1.2. These large plasmids contain the right end of the Ad5 genome with two *Sfi*I sites in place of the E1 region. Like *Alw*NI, *Bst*API, *Dra*III, and *Pfl*MI, the enzyme *Sfi*I recognizes an interrupted palindrome. Both *Sfi*I sites were designed to generate different sticky ends that are compatible with those generated by digesting pE1.1 with *Alw*NI, *Bst*API, *Dra*III, and *Pfl*MI. The ligation products are introduced into *E. coli* either by electroporation or by DNA packaging into phage λ . Using either technique, clones are selected for their resistance to both kanamycin and ampicillin to prevent background of vectors without insert. In addition, the presence of a kanamycin-resistance gene in the recombinant plasmid allows DNA yields that are up to five times higher than those obtained in the presence of ampicillin (not shown).

In the last step of the procedure, the plasmid DNA is linearized with either *Pac*I or *Swa*I and is transfected into a helper cell line such as 293 or 911 (9). The viral plaques appear usually 7–10

days after transfection, sometimes as early as four days.

This directional cloning strategy also enables the *simultaneous* insertion of two expression cassettes into the adenoviral genome, in the E1 and E3 regions (Figure 2). In this setting, the expression cassette destined to the E1 region is cloned into plasmid pE1.2 and thereby linked to a kanamycin resistance gene. The expression cassette destined to the E3 region is cloned into plasmid pE3.1 and thereby linked to a 180-bp λ *cos* site. As with pE1.1, these sequences are surrounded by different sets of *Alw*NI, *Bst*API, *Dra*III, and *Pfl*MI restriction sites, which generate nonpalindromic cohesive ends that are identical within the same set but are incompatible with those generated in another set. After digestion of the intermediate vectors with one of these enzymes, the DNA fragments of interest are cloned into the adenoviral plasmid AdenoQuick13.1. This plasmid, derived from AdenoQuick1.2, contains four *Sfi*I sites (two in the E1 region and two in the E3 region), which generate sticky ends compatible with those generated by digesting, respectively, pE1.2 and pE3.1 with *Alw*NI, *Bst*API, *Dra*III, or *Pfl*MI. The ligation products are introduced into *E. coli* preferentially via packaging into phage λ , and the recombinant clones are selected in the presence of both ampicillin and kanamycin. This procedure guarantees the presence of both expression cassettes in the recombinant plasmid. The subsequent steps (plasmid purification and linearization and helper cells transfection) are performed as described above.

Examples

To illustrate the efficiency of the method, we constructed two viruses, one expressing *Renilla* luciferase in the E1 region and the other expressing both *Renilla* and firefly luciferases, respectively, in the E1 and E3 regions. A cassette expressing *Renilla* luciferase was inserted into the multiple cloning sites of pE1.1 and pE1.2, and a cassette expressing firefly luciferase was inserted into the multiple cloning site of pE3.1. The three resulting plasmids were digested with *Alw*NI. The pE1.1-derived fragment expressing *Renilla* luciferase

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was ligated with *Sfi*I-digested AdenoQuick13.1. The pE1.2-derived fragment containing the *Renilla* luciferase expression cassette and the pE3.1-derived fragment containing the firefly luciferase gene were ligated with *Sfi*I-di-

gested AdenoQuick13.1. The ligation mixtures were introduced into *E. coli* Top10, either by electroporation or via packaging into phage λ . In each transformation experiment, several hundred clones were obtained. The presence and

orientation of the expression cassettes in the recombinant plasmids were verified by restriction analysis. As shown in Table 1, the efficiencies obtained for both single and double insertion experiments (two- or four-fragment ligations)

Figure 2. Strategy for construction of E1- and E3-substituted adenoviral vectors. Refer to Figure 1 for the symbols and the text for the details of the strategy.

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were greater than 99% when the λ packaging extract was used. More than 80% of the E1-substituted clones obtained by electroporation were correct.

For each construct, the plasmid DNAs from four clones were purified, linearized with *PacI*, and transfected into 293 cells. An average of nine plaques/6-cm dish was obtained, the first plaques being visible five days after transfection for the E1-substituted virus and 6 days after transfection for the double recombinant virus. These plaques were further purified and amplified. Titers of 1.0×10^4 and 3.4×10^3 particles/cell were obtained for the single and double recombinant viruses, respectively, compared to 1.4×10^4 particles/cell obtained with wild-type Ad5 virus grown in the same conditions. The identities of the viruses were confirmed by assaying extracts of infected A549 cells for luciferase activity and by restriction analysis of the viral

Table 1. Cloning Efficiencies Obtained in the Construction of Adenoviral Plasmids Containing One Insert in the E1 Region or Two Inserts, Respectively, in the E1 and E3 Regions

		Single Insertion in E1 (Two-Fragment Ligation)	Double Insertion in E1 and E3 (Four-Fragment Ligation)
<i>E. coli</i> Transformation	λ	174/174 (100%)	140/142 (99%)
	e ⁻	35/42 (83%)	2/24 (8%)

The products of ligation between the fragments obtained by digesting the shuttle plasmids and the *SfiI*-digested AdenoQuick plasmids were introduced into *E. coli* either via packaging into phage λ or by electroporation (e⁻). The ratios indicate the number of correct clones obtained, divided by the number of clones analyzed, and are expressed as percentages in bold. The figures represent the sum of several experiments.

DNAs. To test the reproducibility and the efficiency of the method, we have so far constructed more than 20 recombinant adenoviruses, which correctly express the transgenes of interest.

DISCUSSION

Several methods have been designed to construct recombinant adenoviruses by first reconstituting the entire se-

quence of the desired virus in *E. coli* and then transfecting the recombinant plasmid into helper cells (5–8,10–13,16). These methods appear to be preferable, especially when viruses expressing toxic genes need to be constructed (17). However, manipulating large plasmids in *E. coli* is difficult and therefore re-buffing for inexperienced researchers.

With this in mind, we have designed the AdenoQuick system, a set of plasmids that has the following advantages compared to other methods based on the reconstitution of the genome of the desired recombinant virus in *E. coli*.

First, no homologous DNA recombination event is necessary. Techniques that rely on DNA recombination in *E. coli* are more time consuming because the recombination between two plasmids is performed in a *recA*⁺ strain such as BJ5183 and candidate plasmids must be transferred to a *recA endA* strain for better characterization and large-scale purification (5,6,11). In contrast, the present cloning system allows one to construct adenoviral plasmids directly in *recA endA* strains such as DH5 α , XL-1 Blue, or Top10, thereby saving a substantial amount of work.

Second, our cloning system is very efficient. Indeed, each expression cassette is linked to at least one positive-selection marker (Kan^r or λ *cos* site) before being transferred into the large AdenoQuick plasmids. This procedure, which guarantees the presence of the expression cassette(s) in the adenoviral genome, prevents the background of vectors lacking insert(s), thereby eliminating additional counter-selective steps such as a digestion of the ligation products before *E. coli* transformation (13). The correct orientation of the transgene in the adenoviral vectors is ensured by the use of a specific set of restriction enzymes that allow for directional cloning. No treatment of the DNA fragments before ligation is required, as in previously published methods (8,12,16). Our technique generally produces hundreds of *E. coli* clones per experiment, which are found to be correct with greater than 99% efficiency when the phage λ approach is used. This reduces the number of clones to be analyzed, thereby saving a significant amount of work. High plasmid yields are also obtained (up to 3 μ g

cosmid DNA/mL bacterial culture).

Third, our method is very versatile. The transfer of the expression cassette(s) from the shuttle vector(s) to the adenoviral plasmids can be performed either via packaging into phage λ or by electroporation, allowing each investigator to choose a method according to his or her own experimental strengths. Four different enzymes are available to excise the expression cassettes from the intermediate vectors, and unlike most of previously described techniques (5,7,11,13), two rare-cutting enzymes are available to linearize the cosmid before transfection into 293 cells. This method is therefore likely to be useful in a very large number of applications.

Finally, our method is unique in that it allows the simultaneous introduction of two expression cassettes in the E1 and E3 regions of the adenoviral genome, in a single ligation reaction and with a very high efficiency (Table 1). This is in contrast with previously published methods that introduce both transgenes sequentially (8,12) or require agarose gel purification of large DNA fragments (7). This rapid technique will allow further characterization and a more widespread use of E1- and E3-substituted recombinant adenoviral vectors.

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