The Admid System: Generation of Recombinant Adenoviruses by Tn7-Mediated Transposition in *E. coli*

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ABSTRACT

A new system has been developed for generating recombinant adenoviruses by Tn7-mediated transposition in *E. coli*. Low copy number *E. coli* plasmids containing a full-length adenoviral genome with *lacZatt*-Tn7 replacing E1 have been constructed. The adenovirus plasmid or *admid*, as well as high copy number progenitors, were stably maintained in *E. coli* strain DH10B. Several transfer vectors containing a mammalian expression cassette flanked by Tn7R and Tn7L were used as donors to transpose the mini-Tn7 into the E1 region of the adenoviral genome. Transposed recombinant *admid*s are readily identified by their β -galactosidase phenotype. Transfection of *admid* DNA into producer cells resulted in the efficient production of infectious adenovirus. This easy-to-use, efficient system generates pure, clonal stocks of recombinant adenovirus without successive rounds of plaque purification.

INTRODUCTION

Adenoviruses (Ads) are 36-kb, linear, dsDNA viruses that have been widely used for gene therapy, functional genetics, vaccines and protein production (16,19,20,24,26). Most Ad vectors are based on the Ad type 5 (Ad5) viral backbone in which an expression cassette replaces the E1 and/or E3 region. Such E1⁻, E3⁻ viruses are attractive vectors because they: (i) can accept about 8 kb exogenous DNA, (ii) can be produced at very high titer in cells expressing E1 and (iii) are replication defective. Other attractive features of Ad vectors include high-efficiency infection of a wide range of both dividing and quiescent cell types, high-level gene expression and historical association with mild, self-limiting pathologies in humans (4,16,19,20,24,26). However, for gene therapy applications, recent clinical results suggest the toxicity of high-level recombinant Ad infection has been underestimated (21). The increasing numbers of genes discovered by the human genome project and the expanding field of gene therapy are driving the need for more efficient systems to generate recombinant Ad vectors to facilitate rapid, high-throughput functional and therapeutic analysis.

Because of their complex genome, manipulation of Ad vectors to produce recombinant viruses has been difficult. Traditional methods of making recombinant Ads use homologous recombination between two transfected DNAs in Ad-producing cell lines (4). The homologous recombination method is inefficient, time-consuming and subject to contamination with replication-competent virus. Some newer methods of

generating recombinant Ad vectors in yeast and *E. coli* have been reported (7,8,14,16). These methods shift the homologous recombination step from eukaryotic packaging cells into either yeast or *E. coli*. Another recent method (25) uses rare-cutting, intron-encoded endonucleases to insert transgenes into a plasmid bearing a full-length Ad genome. These newer methods allow the true cloning of the recombinant viral genome and remove the need for clonal selection with repeated rounds of plaque purification. A method using the Cre-loxP system to generate recombinant Ad by in vitro recombination has also been reported (1). This method does not allow cloning in *E. coli*, but the recombination results in only one molecule that can produce clonal Ads.

A method that has been used to construct recombinant baculoviruses in *E. coli* by Tn7-mediated, site-specific transposition (22) has proven to be very efficient for generating recombinant baculoviruses. We have developed a Tn7-based transposition system for generating recombinant Ads in *E. coli*. A low copy number adenovirus plasmid (*admid*) containing a full-length Ad genome with *lacZatt*Tn7 replacing E1 has been constructed. The *admid* system is easy, rapid and efficient, accommodates DNA inserts up to 5.8 kb in the current E1⁻, E3⁻ deleted vectors and generates truly clonal viruses. This system is also easily adapted to allow subcloning into a single universal transfer vector that can be used to transpose genes into any of several different expression or viral vector systems, thus facilitating cost-effective subcloning into Ad, baculoviral and *E. coli* vectors (M.W., personal communication).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Cell Lines and Viral DNA

DH10B and DH10Bac competent cells and pFASTBAC1 were obtained from Life Technologies (Rockville, MD, USA). The plasmid pMON7124 (3) was isolated from DH10Bac cells. BJ5183 (13) was provided by D. Hanahan and pGL3-basic was purchased from Promega (Madison, WI, USA). The tetracycline-regulatable promoter (TetOp) and tetracycline-controlled transactivator (tTA) were obtained from H. Bujard (10). P. Fasani-Ghersa (Serono, Geneva, Switzerland) constructed and provided AdtTA. Other reagents include pFOS1 (18), IN340 viral DNA (15), HEK 293 cells (11) and IMR-90 cells (23).

General Methods

Competent cells were prepared by the CaCl_2 method and stored frozen (2). Plasmid minipreps were prepared using Wizard® minipreps (Promega); plasmids larger than 20 kb were eluted from

the minicolumns with distilled water heated to 80°C. For large-scale isolation, 500–1000-mL cultures were grown in LB medium, and the admids were isolated using plasmid maxi kits following the procedure recommended for low copy number plasmids (Qiagen, Valencia, CA, USA). LB agar was supplemented with 100 $\mu\text{g/mL}$ ampicillin (Ap), 15 $\mu\text{g/mL}$ tetracycline (Tc), 20 $\mu\text{g/mL}$ chloramphenicol (Cm), 300 $\mu\text{g/mL}$ Bluo-gal (Life Technologies) and 40 $\mu\text{g/mL}$ isopropylthio- β -galactoside (IPTG) as indicated. PCR was performed with AmpliTaq® (PE Biosystems, Foster City, CA, USA) using standard techniques (2).

Construction of Admid Homing Vectors and Transfer Vectors

The admid vector was constructed in a multistep procedure from IN340 viral DNA, bMON14272 and pFOS1. The final admid contains an E1^- , E3^- Ad genome, the low copy number F' origin and Cm-resistance gene (Figure 1). The *lacZattTn7* (generated by PCR of DH10Bac DNA with primers priCR1

Figure 1. Diagram of admid vector pCR220. The shaded area represents Ad5 sequences and the hatched box indicates the *lacZattTn7*, which replaces approximately 3 kb from the Ad5 E1 region. Indicated are the F' origin of replication (open box) and Cm^R marker (open arrow). An earlier vector, pCR213, has the same Ad genome with a 2.9-kb *PacI* (left end of Ad sequences), *SwaI* (right end of Ad sequences) fragment containing the ColE1 origin and β -lactamase gene replacing the 6.5-kb *PacI* fragment of pCR220.

Research Report

Figure 2. Diagram of transfer vectors. The ColE1 origin, Ap^R marker, Tn7R and Tn7L, are derived from the 3.1-kb *SacII*, *BclI* fragment of pFASTBAC1. (A) The 1.0-kb CMV expression cassette has a multiple cloning site (MCS) between the simian virus 40 (SV40) splice and polyadenylation signals. (B) Vectors with the Tc-regulatable promoter were made by replacing the CMV promoter with the 474-bp *XhoI*, *XbaI* fragment from pUHG10-3 (9). Transfer vectors containing the luciferase reporter gene, pCR219 and pCR232, were made by inserting the 1.7-kb *HindIII*, *XbaI* fragment of pGL3-basic into the MCS of the CMV and TetOp vectors, respectively. Legend: Open boxes, left and right ends of Tn7; hatched filled arrow, Ap^R gene; small and large boxes with left slant, the splice site and polyadenylation signal from SV40, respectively; shaded arrow, CMV promoter; solid arrow, Tc-regulated promoter; filled box MCS-pCR243 and pCR247:*PstI/KpnI/NotI/HindIII/NruI/BamHI*; pCR244 and pCR248: *PstI/KpnI/BamHI/NruI/HindIII/NotI*. (The *KpnI* site is not unique in the TetOp vectors because of the presence of a *KpnI* site in the TetOp promoter.)

5'-GGTCGAGCGTCTTCGAAGCGC-3' and priCR2 5'-CCGTCTTCGAAC-CAATCAGCAAACC-3') replaces about 3 kb of the E1A/E1B region of Ad5 (bases 356–3329 deleted; GenBank[®] accession no. M73260). The final construct, pCR220, is maintained as a low copy number plasmid in *E. coli*, and produces a β-gal⁺ phenotype on media containing Bluo-gal and IPTG. Restriction of pCR220 with *PacI* produces two linear DNA fragments. One fragment is essentially an E1[−], E3[−] recombinant Ad genome with *lacZattTn7* replacing E1. The other fragment carries the DNA needed for replication and selection in *E. coli*.

Although functionally deleted for E3, the E3 mutation in pCR220 is derived from *dl309*, in which the deleted Ad5 E3 DNA was replaced with heterologous DNA, which results in little increase in the cloning capacity (5). To accommodate larger insert sizes, it was desirable to remake the E3 mutation as a physical deletion of DNA. This was accomplished using standard DNA cloning techniques to replace the *dl309*-derived E3 mutation with an E3 mutation, which was made by deleting the

1.9-kb *XbaI* fragment from wild-type Ad5 to create pCR249. The Ad genomes of pCR249 and pCR220 are 32.0 kb and 33.8 kb, respectively, allowing insertions up to 5.8 kb in pCR249 and 4.0 kb in pCR220 without exceeding the Ad packaging capacity (6).

Plasmid transfer vectors containing mini-Tn7 elements (Figure 2) were constructed using standard cloning techniques. Further details on the constructions of homing and transfer vectors are available from the authors.

Construction of *E. coli* Strains DH10B (Admid220) and DH10B (Admid249)

DH10B competent cells were transformed with extrachromosomal DNA isolated from DH10Bac and selected on Tc. Tc^R, Ap^S kanamycin-sensitive colonies containing pMON7124 were isolated. Competent DH10B (pMON-7124) cells were transformed with either pCR220 or pCR249. The transformants were plated on LB agar + Tc + Cm + Bluo-gal + IPTG and were designated DH10B (Admid220) and DH10B (Admid249), respectively.

Table 1. Yield of Recombinant Ad from 293 Transfection

DNA	Treatment	Location of restriction enzyme site ^a	FFU ^b
pCR220::Tn219	<i>PacI</i>	left, right	5.7×10^7
pCR220::Tn219/ pMON7124/pCR219	<i>PacI</i>	left, right	7.7×10^5
pCR220::Tn232	<i>PacI</i>	left, right	3.4×10^7
pCR220::Tn232/ pMON7124/pCR232	<i>PacI</i>	left, right	8.5×10^5
pCR213	none	none	7.6×10^4
pCR213	<i>PacI</i>	left	6.8×10^4
pCR213	<i>SwaI</i>	right	2.7×10^7
pCR213	<i>PacI</i> , <i>SwaI</i>	left, right	1.1×10^7

^arelative to the ends of the Ad genome

^b60-mm dishes of 293 cells were transfected with 2 μ g DNA using 40 μ g LIPOFECTAMINE; five days later, monolayers were scraped from the dishes in 4 mL media, the suspension was frozen and thawed three times and titered on 293 monolayers.

Transposition of Mini-Tn7 Elements from Transfer Vectors into Admids

Transposition was performed by transforming 80 μ L competent DH10B (Admid220) with 10 ng uncut transfer vector. The competent cells were mixed with the transfer vector and incubated for 20 min on ice. After a 45-s heat shock at 42°C and a subsequent 2-min incubation on ice, 900 μ L SOC media (Life Technologies) were added, and the cells were grown for 4 h at 37°C with aeration. The cultures were then plated on LB agar + Cm + Tc + Ap + Bluo-gal + IPTG and grown for 24 h at 37°C. The β -gal⁻ transformants were verified as transposition products by PCR (one primer binding to the Ad sequences of the admid, the other primer binding to an sequence on the mini-Tn7 of the transfer vector). Some of the transposed constructs were further verified using restriction enzyme digests after segre-

Research Report

gating the low copy number recombinant admid from the high copy number pMON7124 helper plasmid and the transfer vector by CaCl_2 transformation.

Production and Purification of Ad

One day after plating about 2×10^6 HEK 293 cells per 60-mm dish, the cells were transfected using 2 μg plasmid DNA and 40 μg LIPOFECTAMINETM (Life Technologies). The DNA/LIPOFECTAMINE complex was incubated with the cells for 6 h in serum-free media before diluting the transfection mixture with an equal volume of media containing 4% bovine calf serum. The cells were harvested after six days, usually before any cytopathic effect was observed. The cells were frozen and thawed three times, the cell debris pelleted and the virus-containing supernatant was titered and amplified.

The virus was titered by infecting confluent plates of HEK 293 cells with varying virus dilutions for 1 h. Eighteen

Figure 3. Luciferase expression from recombinant Ads. (A) diagram of recombinant Ads. Viruses that were made by the admid system have *lacZ* and Tn7 sequences (open symbols) flanking the luciferase expression cassette. Hatched boxes: Ad5 sequences; open arrow: *lacZattTn7*; open boxes: Tn7L and Tn7R; filled arrow: luciferase coding sequence; shaded arrow: CMV immediate early promoter; hatched arrow: Tc-regulated promoter; boxes with left slant fill: SV40 splice (small box) and SV40 polyadenylation signal (large box). (B). Luciferase expression 48 h after infecting IMR-90 cells at an MOI of 100 (50 MOI luciferase virus + 50 MOI either Adnull or AdtTA virus). (C) Tc-regulated expression 48 h after infecting IMR-90 cells at an MOI of 200 or 20 (half of virus contained luciferase gene and half of virus either Adnull or AdtTA). When indicated, Tc was added one day before viral infection.

hours later, the cells were fixed with 90% methanol, and the number of infected cells was determined by fluorescent microscopy [primary antibody: mouse Ab to Ad (Biodesign International, Kennebunk, ME, USA); secondary antibody: FITC-labeled goat anti-mouse antibody (ICN, Aurora, OH, USA)]. Amplified virus was extracted with Freon and purified by banding in CsCl gradients (12). The purified virus was dialyzed against 10 mM Tris HCl (pH 8.0), 10 mM MgCl₂, 150 mM NaCl, 10% glycerol and stored at -25°C.

Construction of Conventional Control Viruses

The control viruses, Adnull and Ad225, were constructed by the traditional method of homologous recombination in 293 cells (4). The recombinant viruses were identified by PCR analysis of the primary amplification of individual plaques, purified again and then amplified. The viruses were produced and purified as described for the viruses produced using the admid system.

Infection of Cell Lines and Luciferase Assays

IMR-90 cells were plated at 5×10^4 cells/well in 6-well dishes. Two days later, after undergoing two population doublings, triplicate wells were infected with Ad mixtures at the indicated MOI. Each infection contained equal amounts of luciferase virus and either Adnull or AdtTA virus. Two days later, the cells were harvested and assayed for luciferase activity using the luciferase assay system with cell culture lysis reagent as recommended by the supplier (Promega). Luciferase activity was quantitated using a ML3000 luminometer (Dynatech Labs, Chantilly, VA, USA). Where indicated, the cell culture medium was supplemented with Tc starting one day before viral infection.

RESULTS AND DISCUSSION

Construction of Plasmid Vectors

The development of the admid system required the construction of two plasmid components: (i) a low copy

number admid homing vector containing an Ad genome with a *lacZattTn7* site (Figure 1), and (ii) an admid transfer vector with a mini-Tn7 containing an expression cassette appropriate for expression in Ad vectors (Figure 2). The target site for Tn7 insertion, *attTn7*, is located in the *lacZ* gene. Transposition into *attTn7* disrupts the *lacZ* reading frame, resulting in a β -gal⁻ phenotype. The final construct is maintained as a low copy number plasmid in *E. coli* to facilitate screening for the β -gal⁻ phenotype.

The mini-Tn7 transposition cassette within the admid transfer vectors was designed to be as small as possible while providing efficient transposition, screening for transposed admids and transgene expression in mammalian cells. The "bacmid" system described by Luckow et al. (22) incorporates a gentamycin-resistance marker within the mini-Tn7 to aid in the selection of transposed constructs. We have found the antibiotic marker within the Tn7 transposition cassette to be unnecessary; similar transposition efficiencies were obtained whether or not an antibiotic marker within the mini-Tn7 cassette was used to select for the transposed constructs (data not shown). Several mini-Tn7 admid transfer vectors have been constructed (Figure 2). Convenient restriction sites are present in which to insert sequences under the control of the cytomegalovirus (CMV) or Tc-regulatable promoter. These sites may also be used to replace the CMV promoter with any other desired regulatory sequence.

Transposition and Confirmation of Transposition by PCR

The host for transposition was an *E. coli* strain DH10B that contained the plasmid pMON7124 [encoding the *trans*-acting Tn7 transposase genes and Tc-resistance (3)] and an admid (containing the *lacZattTn7* gene). Transposition occurs following the introduction of a transfer vector containing a mini-Tn7 element into the host strain. The transposition and screening for recombinant admids containing a mini-Tn7 expression cassette occurs in a single step that is efficient and easily scored as a β -gal⁻ phenotype on media containing Bluo-gal and IPTG. The percentage of β -gal⁻ colonies obtained appears to

Research Report

vary based on an uncharacterized feature of the competent DH10B admid cells used for the transposition. For example, the nine transpositions performed with the first batch of frozen competent cells were carefully monitored. The transposition efficiency averaged 23%, range 9%–40% β -gal⁺ colonies. Although more recent batches of frozen competent cells have been less efficient, our experience using this system to make over 40 recombinant Ads has been that every transposition experiment has yielded β -gal⁺ colonies. Furthermore, all the β -gal⁺ colonies we have tested represent true transposition products (PCR product using one primer binding to sequences present on the admid and the second primer binding to the transfer vector).

Transfection of HEK 293 Cells and Initial Viral Titers

After clonal isolation of the β -gal⁺ isolates, admid DNA was isolated and restricted with restriction enzyme *PacI*.

Transfection of HEK 293 cells with admid DNA led to efficient production of infectious Ad (Table 1). Higher amounts of virus were produced if the cells were transfected with only the transposed recombinant admid DNA rather than a mixture of DNAs containing admid, pMON7124, and transfer vector. This likely reflects the increased molar amount of admid DNA present in 2 μ g pure admid DNA versus the lesser molar amount of admid DNA in 2 μ g mixed DNA (a low copy number admid and two high copy number plasmids).

Results obtained with pCR213, a predecessor of pCR220 that contains a *PacI* site at the left end and a *SwaI* site at the right end of the Ad genome (see Figure 1 legend), demonstrated that the virus was produced even if the admid DNA was not restricted before transfecting 293 cells. However, approximately 100-fold more virus was obtained from admid DNA that was restricted at either the right or both ends of the adenoviral genome compared to undigested admid DNA or DNA restricted only at the left end of their Ad genome (Table 1). Therefore, in most cases, it will be beneficial to digest the admid with *PacI* before transfection. However, in those rare cases when the expression cassette has a recognition site for *PacI*, the digestion can be omitted if an additional round of amplification is performed before large-scale virus production.

Titers and Expression from Admid Vectors

We have also compared the yield of infectious virus and expression capabilities of E1⁺, E3⁺ Ads generated by conventional and admid methods. Both the traditional method of homologous recombination in 293 cells and the admid method were used to construct viruses expressing luciferase. The CMV-luciferase expression cassette is identical between Ad225 and Ad220::Tn219. The only differences between Ad225 and Ad220::Tn219 are the addition of *lacZ* and Tn7 sequences flanking both sides of the CMV-luciferase expression cassette in Ad220::Tn219 (Figure 3A). When equivalent numbers of 293 cells were infected and the virus was purified, similar amounts of virus were obtained [Ad225, 9.2×10^{10} focus-form-

ing units (FFU); Ad220::Tn219, 6.5×10^{10} ; Ad249::Tn219, 6.6×10^{10} FFU]. Neither the titer nor the final yield of virus was altered by the *lacZ* or Tn7 sequences of Ad220::Tn219 or the alternative E3 deletion of pCR249.

To test the expression levels obtained from each virus, IMR-90 cells were infected with recombinant Ad, and the luciferase activity was quantitated (Figure 3B). A comparison of the luciferase activity from cells infected with either Ad225 or Ad220::Tn219 shows the same luciferase expression from viruses made by either method. This indicates that the flanking *lacZ* and Tn7 sequences do not affect the expression of the CMV promoter. Essentially equal expression was also obtained from Ad249::Tn219 indicating the alternative E3 deletion also did not affect luciferase expression.

Ads for Efficient Functional Analysis

The ability to modulate the level of expression of an introduced gene over a broad range should facilitate efficient functional analysis experiments by allowing such studies to be carried out at very high, very low or physiological levels of gene expression with one set of viral reagents. Figure 3C demonstrates the broad range of expression that can be obtained using the Tc-regulated gene expression system (10) in Ad vectors. Little to no expression is detected in cells infected with the tetOp-luciferase virus (Ad220::Tn232) in the absence of the AdtTA transactivator virus (open symbols). However, when cells are co-infected with both viruses, a large amount of luciferase activity is observed (filled symbols). Also, adding Tc to the cell culture media allowed for the fine-tuning of the absolute level of luciferase expression.

The admid system could also be adapted to carry out efficient protein expression and gene therapy studies. By moving the promoter from the transfer vector onto the admid in such a way that after transposition the transposed gene is regulated by a promoter flanking the *lacZattTn7*, it should be possible to make one transfer vector that could be transposed into a series of admids that contain different promot-

Research Report

ers. Making similar adaptations to bacmids would allow the same "universal" transfer vector to be used to make both Ads and baculoviruses. It should also be possible to generate a wide range of viral vectors with attTn7 sites (e.g., herpes, AAV and retroviral vectors). If the appropriate homing vectors (that is, admids, bacmids, etc.) are made, a "universal" transfer vector could further increase the speed and lower the costs of expressing large amounts of functional protein for purification, for in vitro and in vivo functional analysis or to optimize delivery for gene therapy. Such increases in efficiency will be required to analyze the many genes being discovered by the human genome project and for rapid, cost-effective comparisons among viral delivery systems.

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