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A fast way to generate recombinant adenoviruses: a high-frequency-recombination system

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The use of adenovirus for gene transfer has increased dramatically. One of the most time-consuming steps in any project involving adenovirus is to generate a unique recombinant adenovirus. This report provides a way to generate recombinant adenoviruses with significant reduced time and labor and a high rate of success. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 198–202.

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Introduction

The application of adenoviral vectors for gene transfer has become widely used for gene therapy [2,9,10,16] and vaccine development [5]. The critical and time-limiting step is the generation of a unique recombinant adenovirus. The current methods available to generate a recombinant adenovirus are time-consuming and result in a poor yield of the intended recombinant adenovirus. A typical system to produce a replication-defective recombinant adenovirus consists two parts: one is an E1-deleted adenoviral shuttle vector into which the gene of interest has been subcloned; the other is an adenoviral genome provider which can be either a portion of the adenovirus genome (eg the large *Cla*I fragment) or a plasmid containing the adenoviral genome. These two parts are cotransfected into human embryonic kidney 293 cells [6] and the resultant adenoviral plaques are screened for the recombinant adenovirus. Common problems that occur in the current available systems include: (a) a long waiting period for plaque formation; (b) no plaques at all when using plasmid-containing adenoviral genome; or (c) a high background of non-recombinant adenovirus in the pool of plaques when using *Cla*I fragment of adenovirus. As a consequence, the time and labor for generating a recombinant adenovirus are increased. We studied different combinations of adenoviral shuttle vector and adenoviral genome provider and established the best homologous recombination system that gave the highest frequency of *in vivo* recombination and a shorter lag time for plaque formation.

E. coli β-galactosidase (*lacZ*) has been used as a marker gene for studying gene transfer efficiency [4,14] and evaluating tissue-specific promoter activity and specificity [1,7,15,17]. Very recently it has been shown that *lacZ* gene itself can be used directly as a therapeutic prodrug enzyme gene in combination with LacZ prodrug [12]. Therefore the generation of various *lacZ*-adenovirus has become routine and in increasing demand. A method to quickly identify the recombinant *lacZ*-adenovirus was developed by using plaque overlay containing LacZ substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The combination of using this high-frequency-recombination system and X-Gal staining results in the rapid generation and identification of *lacZ*-adenovirus.

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Materials and methods

Cell culture and medium

Human embryonic kidney 293 cells were purchased from ATCC (Rockville, MD, USA). Dulbecco's modified Eagle medium (D-MEM) was purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT, USA). Cells were grown in D-MEM with 10% FBS at 37°C in 5% CO_2.

Cotransfection of kidney 293 cells with adenoviral shuttle vector and Ad5 genome providers

Adenoviral shuttle vectors pAvs6a and adenovirus Ad5 dl-327 were obtained from Genetic Therapy (Gaithersburg, MD, USA). Adenoviral shuttle vector pCA14, Ad5 genome containing plasmids pJM17 [13] and pBHG10 [3] were purchased from Microbix Biosystems (Toronto, Ontario, Canada).

Kidney 293 cells were grown in a 100-mm culture dish until 80% confluent. For each adenoviral construct, one 100-mm dish of kidney 293 cells was cotransfected with either 15 μg adenoviral shuttle vector and 5 μg plasmid containing Ad5 genome, or 5 μg adenoviral shutter vector and 2.5 μg *Cla*I fragment of adenovirus dl-327, by the calcium phosphate method [8]. After overnight incubation at 37°C, the cells were washed twice with PBS, and covered with overlay mixture: 1:1 of 2% Seaplaque agarose (FMC, Rockland, ME, USA) and 2 × plaque mix (2 × MEM, 15% FBS, 4 mM glutamine, 1 × Pen/Strep, and 0.25 μg ml^{-1} of fungizone, all from Gibco BRL). The cells were overlaid with fresh overlay mixture every 5–6 days until the plaques were ready to be picked.

Direct plaque screening by polymerase chain reaction (PCR)

Plaques were picked and screened by the direct plaque-screening method using DNA extracted from plaques as

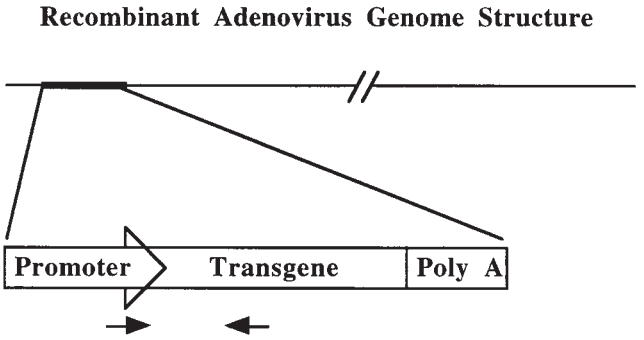

Figure 1 Schematic drawing of the recombinant adenovirus genome structure and the PCR primer locations. The E1A region of adenovirus Ad5 was replaced with transgene expression cassette to generate replication-defective recombinant adenovirus. The transgene expression cassette is shown. Arrows represent two primers used for PCR screening for the recombinant structure.

described [11]. A pair of primers with one specific for promoter and the other for transgene was used for PCR (Figure 1).

X-Gal overlay of plaques

The standard plaque overlay was performed on kidney 293 cells as described above. After the plaques reached about 2-mm diameter, another overlay mixture containing 0.6 mg ml^{-1} of X-Gal (Gibco BRL) was applied to the plaques. The cells were incubated at 37°C overnight or until the positive plaques exhibited a blue color (in cases where tissue-specific promoters were used to drive *lacZ* expression, longer incubation of X-Gal-containing plates was necessary and a microscope might be needed to observe the blue plaques due to the weak and leaky promoter expression in kidney 293 cells). Several blue plaques were picked for direct plaque-screening for confirmation of the construct.

Results

High-frequency-recombination system to generate recombinant adenovirus

To optimize the homologous recombination system resulting in a higher frequency of *in vivo* recombination and a shorter lag time for plaque formation, different combinations of adenoviral shuttle vector and adenoviral genome provider were tried including the combinations suggested by the manufacturers, that is, pAvs6a with *Cla*I fragment of adenovirus dl-327 and pCA14 with pJM17 or pBHG10. To eliminate the effect of the transgene itself, the same cDNA gene was subcloned into both adenoviral shuttle vectors pAvs6a and pCA14 respectively. Table 1 shows that any combination of *Cla*I fragment of adenovirus dl-327, though with a relatively short lag time for plaque

Table 1 Combinations of adenoviral shuttle vector and adenovirus Ad5 genome provider

Combination	Lag time of plaque formation	Frequency of *in vivo* recombination
pAvs6a-derived + pJM17	11 days	high
pAvs6a-derived + *Cla*I fragment	10 days	low, with high background plaques
pCA14-derived + pJM17	no plaques[a]	low
pCA14-derived + pBHG10	no plaques[a]	low
pCA14-derived + *Cla*I fragment	10 days	low, with high background plaques

The same cDNA gene of interest was subcloned into either pAvs6a (pAvs6a-derived) or pCA14 (pCA14-derived) adenoviral shuttle vector. Each combination shown above was cotransfected into one 100-mm dish of kidney 293 cells (see Materials and Methods for details). At least three independent trials were conducted for each result.
[a]Up to 5 weeks no plaques appeared.

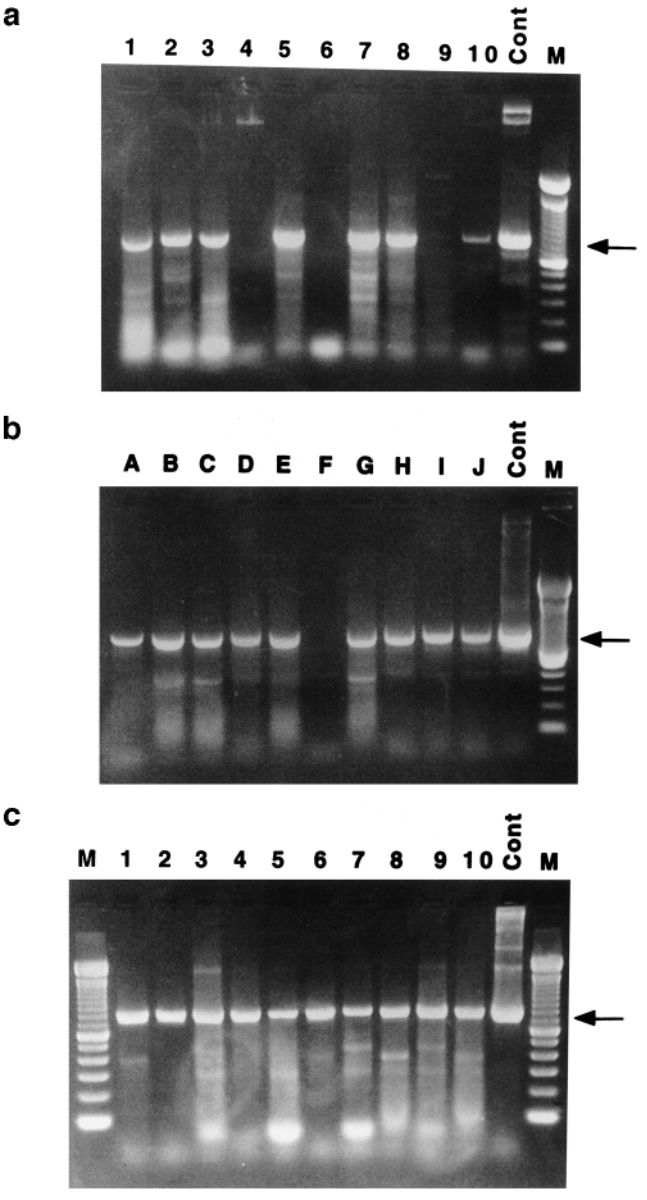

Figure 2 Direct plaque-screening by PCR for recombinant adenovirus. The viral DNA extracted from plaques was screened by PCR using primers specific to the transgene (see Figure 1). Shown are the PCR results of 10 plaques each from the first round (a), second round (b), and third round (c) of plaque purification. Twenty microliters of each 50-μl PCR reaction mixture were loaded on a 2% agarose gel and eletrophoresed. A 799-bp signal (arrow) of the transgene-specific PCR product indicated that the plaque was a positive recombinant adenovirus. Recombinant adenoviral shuttle vector plasmid was used as a positive control (Cont) for PCR. Markers (M) were a 100-bp DNA ladder.

formation, gave a high level of background plaques due to the contamination of uncut whole adenovirus Ad5 genome. Plasmid containing adenovirus Ad5 genome gave fewer *in vivo* recombinants with its own shuttle vector pCA14. In this case, when one dish of kidney 293 cells was cotransfected, there was no plaque formation up to 5 weeks from the combination of pCA14 with either pJM17 or pBHG10 (Table 1). A better system was the combination of pAvs6a and pJM17, which resulted in a high frequency of recombinant adenovirus at a shorter lag time for plaque formation.

Using this system, only one 100-mm dish of kidney 293 cells was needed for each adenoviral construct to produce the intended recombinant adenovirus. The lag time for plaque formation was usually 7–11 days. The resultant plaques appeared with a recombinant frequency (positive plaques/total plaques screened) between 60–100% for the first round of plaques (formed by cotransfection), 80–100% for the second round of plaque purification, and 100% by the third round of plaque purification. Figure 2 shows the PCR results obtained from DNA in the plaques generated from different rounds of plaque purification by direct plaque-screening [11], with one primer specific for RSV promoter and the other one for β-lactamase cDNA gene for recombinant adenovirus expressing β-lactamase. Ten plaques were randomly picked in the first round and the result showed that seven out of 10 of these plaques were positive (Figure 2a). Clone 3 was chosen and diluted and subjected to a second round of plaque purification. Ten plaques were randomly picked from the second round of plaques and nine out of 10 plaques were positive (Figure 2b). Plaque H was chosen for the third round of plaque purification. Ten plaques were randomly picked from the third round of plaques and all were positive (Figure 2c), indicating a homogenous population of recombinant adenovirus. By using this high-frequency-recombination system, we were able to routinely generate several recombinant adenoviruses simultaneously in a short period of time.

X-Gal overlay for lacZ-adenovirus

The same principle that uses X-Gal to selectively pick blue colonies of bacteria, which harbor *lacZ*-containing plasmids, was applied to select the recombinant *lacZ*-adenovirus. X-Gal was added to the standard plaque overlay mixture to identify blue plaques which were likely to contain *lacZ*-adenovirus. Figure 3 represents part of a blue plaque which localized the recombinant *lacZ*-adenoviruses in a X-Gal-stained kidney 293-cell dish. The blue plaques were then selected for direct plaque-screening by PCR to confirm their *lacZ*-containing status. With the assistance of blue color, the plaques picked were clearly identified and up to 100% positive plaques for *lacZ*-adenovirus were identified during the first round.

Discussion

In this study we report a fast and simple way to generate recombinant adenoviruses by using a high-frequency-recombination system. By combining this system and the fast screening method described previously [11], the time and labor for generating a replication-defective recombinant adenovirus may be significantly reduced with a high likelihood of success.

Depending on the original shuttle vector, the subcloned transgene, and the method chosen to generate recombinant adenoviruses, the *in vivo* recombination frequency in kid-

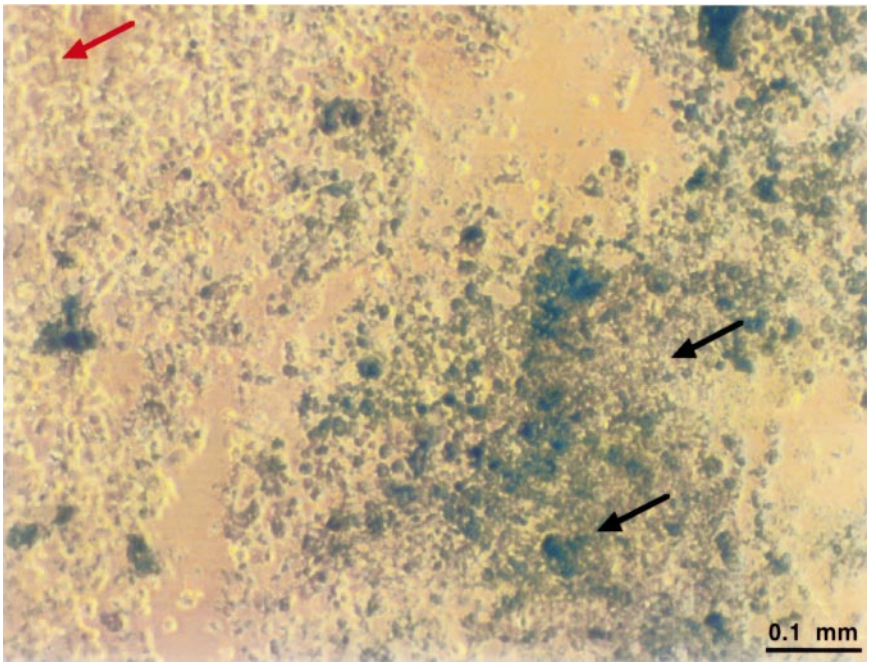

Figure 3 Blue plaque of lacZ-adenovirus on X-Gal-overlay plate. Plaques were overlaid with a mixture containing 0.6 mg ml^{-1} of X-Gal and incubated for 24 h at 37°C. The blue plaque indicated that they were *lacZ*-adenoviruses. Only a portion of the whole plaque is shown in this figure (a scale bar of 0.1 mm is illustrated) and the entire figure is part of a plaque except the left upper corner area that are the uninfected kidney 293 cells (red arrow). The cells show blue color and within the plaque had the round shape, the typical morphology of cells with cytopathic effect (black arrows).

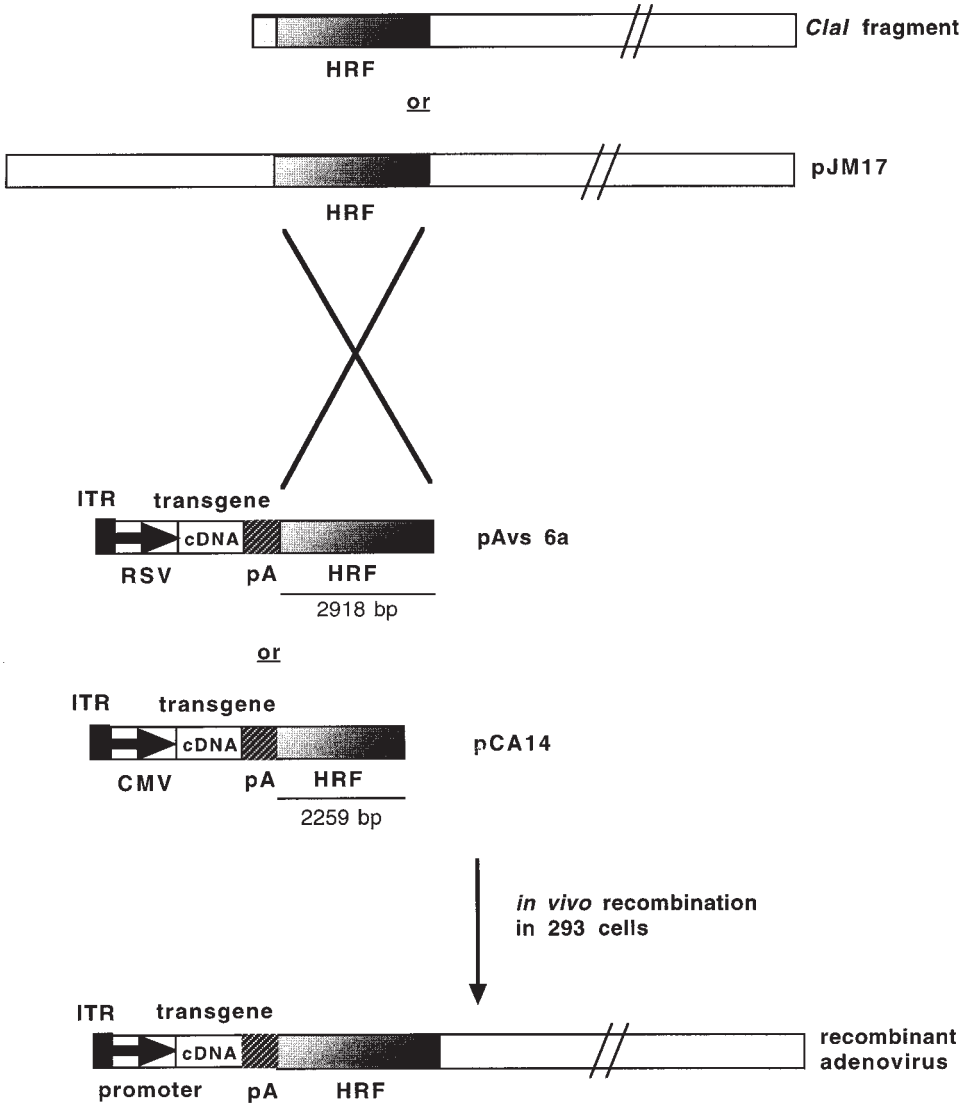

Figure 4 Schematic diagram of generation of recombinant adenovirus. *In vivo* recombination in kidney 293 cells between adenovirus genome (either the large *Cla*I fragment of adenovirus Ad5 dl-327 or pJM17 plasmid) and adenoviral shuttle vector (either pAvs6a or pCA14) will generate the final recombinant adenovirus. pCA14's HRF consists of 2259 bp corresponding to 3534–5792 of the adenovirus Ad5 genome sequences and pAvs 6a's HRF consists of 2918 bp corresponding to 3329–6246 of the adenovirus Ad5 genome sequences. CMV, cytomegalovirus promoter; HRF, adenovirus Ad5 homologous recombination fragment region; ITR, inverted terminal repeats packaging sequence; pA, SV40 poly A region; RSV, Rous sarcoma virus promoter.

ney 293 cells and the lag time to observe the forming plaques were quite different. Based on our experience and that of others, all the available systems have problems of the relatively long lag time for plaque formation and low *in vivo* recombination frequency. The system using the large *Cla*I fragment of adenoviral genome showed a high level of background plaques owing to difficulty in completing separation of uncut or *Cla*I-cut adenoviral genome. Consequently, a large number of plaques must be screened. In contrast, the system that uses plasmids containing adenoviral genome such as pJM17 and pBHG10 results in a much lower level of *in vivo* recombination. It requires multiple dishes of kidney 293 cells with cotransfection to obtain one adenoviral construct. Moreover, the lag time for plaque formation was long—3 weeks or longer. The high background plaque-negative results from a large amount of plaque screening, or fewer or even no plaques from multiple cotransfections, plus the long waiting period can limit the generation of wanted adenoviral recombinations. To establish a better *in vivo* recombination system, we have tried several different combinations of adenoviral shuttle vector and genome provider. The combination of adenoviral shuttle vector pAvs6a and pJM17 gave the best results. The high-frequency-recombination system is better than either of the original systems. By using this new system, one can generate several different adenoviral vectors simultaneously in a short period of time. Interestingly, neither combination of pAvs6a-derived shuttle vector with its own viral genome provider, the large *Cla*I fragment of adenovirus Ad5-dl327, nor the combination of pJM17 with its

own adenoviral shuttle vectors such as pCA14, gave *in vivo* recombination results as satisfactory as that by pAvs6a derived with pJM17.

One explanation of the observed higher recombination between pJM17 and pAvs6a is that pAvs6a has the longer adenovirus Ad5 homologous recombination fragment (HRF) region than that of pCA14. As shown in Figure 4, pAvs6a's HRF has 2918 bp which corresponds to the sequences from 3329 to 6246 of the adenovirus Ad5 genome. In contrast, pCA14's HRF has 2259 bp (corresponding to the sequences from 3534 to 5792 of the adenovirus Ad5 genome), with 205 bp missing at the 5′ end and 454 bp missing at the 3′ end compared to that of pAvs6a's HRF. The extra 659 bp in pAvs6a's HRF region may contribute to the higher frequency of recombination between pAvs6a and pJM17. Early region 1 (E1) coding sequences are from 560 to 3510 of adenovirus Ad5 genome sequences, pCA14 has a complete E1 deletion from 342 to 3524 of adenovirus Ad5 genome sequences, whereas pAvs6a has a deletion from 394 to 3330 of adenovirus Ad5 genome sequences which consist of the entire E1A region and most part of the E1B region. In addition, the inverted terminal repeats (ITR) packaging sequence of pAvs6a contains 392 bp corresponding from 1 to 392 of adenovirus Ad5 genome sequences, whereas pCA14's ITR has only 321 bp corresponding from 21 to 341 of adenovirus Ad5 genome sequences. These differences which mean pAvs6a has more homologous flanking sequences to the adenovirus Ad5 genome may also contribute to the fast and easy recombination between pAvs6a and pJM17. Moreover, pJM17 contains the entire Ad5 DNA molecule with an insertion in the E1 region that exceeds the packaging constraints of the adenovirus capsid [13], therefore pJM17 itself generates very few or no background-negative plaques. In contrast, the large *Cla*I fragment usually generates high background plaques due to the contamination of uncut adenovirus Ad5 genome.

Generation of a *lacZ*-adenovirus has become popular for gene therapy studies. Though identification of the recombinant *lacZ*-adenovirus by staining infected targeted cells with the β-galactosidase substrate X-Gal is possible, one still needs to PCR-amplify the DNA extracted from each of the plaques on kidney 293 cells, and then use a portion of the virus to infect the targeted cells. We have developed a simpler and faster way to identify the *lacZ*-adenovirus by incorporating X-Gal in the overlay mixture. The advantage of this method is that blue plaques that are picked are most likely to contain the recombinant *lacZ*-adenovirus. By the combination of the high-frequency-recombination system and this X-Gal overlay method, several *lacZ*-adenoviruses with different promoters were quickly generated [15,17].

The combination of using pAvs6a and pJM17, or so-called high-frequency-recombination system provides a high likelihood of successful generation of recombinant adenoviruses in a short period of time. By using this system and the direct plaque-screening by PCR [11], at present we can routinely generate several different recombinant adenoviruses simultaneously within a month, starting from subcloning the gene of interest into pAvs6a.

Acknowledgements

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