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Yeast Recombinase FLP Functions Effectively in Human Cells for Construction of Adenovirus Vectors

BioTechniques 29:524-528 (September 2000)

ABSTRACT

We have recently developed a high-efficiency method of constructing adenovirus vectors based on Cre-mediated recombination between two plasmids co-transfected into 293 cells. The simplicity and efficiency of this method should greatly expedite the construction of most recombinant vectors. However, this system would not be suitable for constructing vectors bearing loxP sites elsewhere in the genome because of undesirable Cre-mediated vector rearrangements. To address this, we have developed a similar system using FLP-mediated site-specific recombination for the construction of adenovirus vectors.

INTRODUCTION

Adenoviruses (Ads) are excellent mammalian gene transfer vectors because of their stability, their ability to efficiently infect a wide variety of quiescent and proliferating cell types from various species to direct high-level viral gene expression, the relative ease with which their 36-kb dsDNA genome can be manipulated by molecular biology techniques and the ease by which they can be propagated and purified to high titers. Consequently, Ads are extensively used as vectors for recombinant vaccines, high-level protein production in cultured cells and gene therapy (2,8,10,12).

A popular method for constructing Ad vectors relies on homologous recombination between two plasmids co-transfected into 293 cells (3). In this method, foreign DNA is inserted into a small shuttle plasmid that contains the left end of the Ad genome including the inverted terminal repeat (ITR), the packaging signal and a multiple cloning region in place of E1 for foreign DNA insertion. The second plasmid contains the entire Ad genome modified to be

noninfectious. Foreign DNA is rescued into recombinant viruses by in vivo homologous recombination between overlapping Ad sequences following co-transfection of 293 cells. This method offers several advantages over others for its simplicity, but the efficiency of vector rescue is low because of inefficient homologous recombination.

We recently improved the efficiency of the two-plasmid rescue method 100-fold over the original approach by inserting a loxP site into the plasmids and substituting Cre-mediated for homologous recombination and by replacing the single ITR in the shuttle plasmids with an ITR junction (13,14). The simplicity, efficiency and reliability of this method make it ideal for the routine construction of most Ad vectors. However, this method is incompatible with the construction of vectors that have loxP sites elsewhere in the genome designed, for example, to regulate transgene expression (1,4,7) or to inhibit vector packaging (15).

We addressed this limitation by developing a two-plasmid rescue method based on FLP-mediated site-specific recombination and compared it with the Cre/loxP system. FLP-mediated recombination can now be used as an alternative to Cre-mediated recombination when the latter is unsuitable or undesirable for vector construction.

MATERIALS AND METHODS

The plasmids used in these studies were constructed by standard protocols, prepared by the alkaline lysis method (5) and purified by CsCl-density gradient centrifugation. The FLP expression cassette in pBHGfrtΔE1,3FLP was a generous gift from Volker Sandig, Merck Research Laboratories (West Point, PA, USA). Propagation of 293 cells (9) and co-transfections were performed as described (11). β-galactosidase expression from viral or mock-infected cells was determined by X-Gal staining as described (13).

RESULTS AND DISCUSSION

We recently described a simple and efficient method of constructing Ad

vectors based on Cre-mediated site-specific recombination between two plasmids co-transfected into 293 cells (13,14). Since the use of Cre recombinase may not always be desirable, we developed an alternative method based on site-specific recombination between frt sites catalyzed by FLP recombinase (6) and compared the two systems by rescuing vectors expressing β-galactosidase (Figure 1).

The shuttle plasmids used in this study, pCA35loxΔITR and pCA35frtΔITR, are shown in Figure 2. Essential features include an ITR junction, a packaging signal and a LacZ expression cassette, followed by a loxP site in pCA35loxΔITR or an frt site in pCA35frtΔITR. The Ad genomic plasmids used in this study, pBHGloxΔE1,3Cre and pBHGfrtΔE1,3FLP, have a deletion of the E1 region including the packaging signal, rendering them noninfectious (Figure 2). A loxP or an frt site has been inserted near the 5' end of the pIX gene of pBHGloxΔE1,3Cre and pBHGfrtΔE1,3FLP, respectively, to permit site-specific recombination with the appropriate shuttle plasmid. The Cre or FLP expression cassette has been inserted in pBHGloxΔE1,3Cre and pBHGfrtΔE1,3FLP, respectively, to provide transient recombinase expression and induce site-specific recombination following co-transfection of any Ad5 E1-complementing cell line. The recombinase expression cassettes were inserted into the Ad genomic plasmid so that they are not rescued into the recombinant vector following site-specific recombination (Figure 1) (14).

To compare the efficiencies of Cre- versus FLP-mediated vector rescue 293 cells were co-transfected with the appropriate combination of plasmids, and the numbers of plaques generated were counted (Table 1). As seen from our previous study (13,14), high-efficiency vector rescue was achieved by Cre-mediated recombination following co-transfection of 293 cells with pCA35loxΔITR and pBHGloxΔE1,3Cre as evident by the large numbers of plaques generated (Table 1). Similar numbers of plaques were obtained following co-transfections with pCA35frtΔITR and pBHGfrtΔE1,3FLP, indicating that the efficiencies of Cre- and FLP-mediated vector rescue were comparable. We

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Table 1. Vector Rescue Efficiency by Cre- versus FLP-Mediated Recombination^a

Shuttle plasmid	Ad genomic plasmid	Plaques/dish (Average/dish)	
		Experiment 1	Experiment 2
pCA35loxΔITR	pBHGloxΔE1,3Cre	59, 45, 45 (50)	52, 57, 31, 42 (45.5)
pCA35ftrΔITR	pBHGftrΔE1,3FLP	45, 41, 44, 45 (44)	39, 40, 31, 44 (38.5)

^a60-mm dishes of 293 cells were co-transfected with 2 μg each plasmid, and plaques were counted 10 days after co-transfection.

previously showed that all analyzed plaques that were generated by Cre-mediated recombination had the correct DNA structure and expressed the foreign transgene (13,14). To determine if this was also the case for FLP-mediated vector rescue, 21 plaques were isolated for analysis. All plaques had the expected DNA structure as determined by restriction enzyme analysis of the vector DNA, and all were positive for β-galac-

tosidase expression (data not shown).

Construction of Ad vectors by these approaches offers several advantages over other methods. (i) The method requires only two steps—insertion of the foreign DNA into a small (3–4 kb) shuttle plasmid, followed by co-transfection of 293 cells with an Ad genomic plasmid. (ii) Only plasmid DNA are required as substrates that can be propagated in any routinely used *E. coli*

strain. (iii) Unlike other methods that require linearization of plasmid DNA before transfection, neither the shuttle plasmid nor the genomic plasmid requires restriction endonuclease digestion. This is especially important if the restriction enzyme site used to release the Ad genome is also present in the foreign DNA, thus rendering these methods unsuitable. (iv) Many plaques, which start to appear as early as five days after co-transfection, are obtained to virtually guarantee successful vector rescue. (v) All plaques have the expected DNA structure and express the foreign transgene.

We have now constructed a variety of shuttle plasmids to permit cloning of virtually any foreign DNA up to about 8 kb for Ad vector construction by either Cre- or FLP-mediated recombination. These shuttle plasmids and the Ad genomic plasmids used in these studies can be obtained from Microbix Biosystems (www.microbix.com). We and

Figure 1. Construction of Ad vectors by Cre- or FLP-mediated site-specific recombination following co-transfection of 293 cells with Ad genomic plasmid (pBHGloxΔE1,3Cre or pBHGftrΔE1,3FLP) and shuttle plasmid (pCA35loxΔITR or pCA35ftrΔITR). Ad and bacterial plasmid sequences are represented by thick and thin lines, respectively, and loxP or ftr sites are represented by arrowhead. LoxP and ftr are the target sites for Cre and FLP recombinase, respectively.

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Figure 2. Plasmids used to rescue Ad vectors expressing β -galactosidase. The shuttle plasmids pCA35lox Δ ITR and pCA35ftr Δ ITR contain an ITR junction, a packaging signal (ψ), a LacZ expression cassette and a loxP site in the former and an ftr site in the latter. The noninfectious Ad genomic plasmid pBHGllox Δ E1,3Cre (14) contains the entire Ad5 genome except for deletions of E1 including ψ and E3, a loxP site 5' of the pIX gene and a Cre expression cassette in place of E1. The plasmid pBHGftr Δ E1,3FLP is identical to pBHGllox Δ E1,3Cre except for replacement of the loxP with an ftr site and the Cre with the FLP expression cassette. Thick and thin lines represent Ad and bacterial plasmid sequences, respectively. Arrowhead represents loxP or ftr sites, and small black arrows represent ITRs.

others at McMaster University have now rescued a wide variety of expression cassettes into Ad vectors by these methods with efficiencies comparable to those reported here for the LacZ cassette. The possibility of choosing between two different recombinases, both functioning with high efficiency, allows maximum flexibility in the design of recombinant Ad vectors.

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This work was supported by grants from the National Institutes of Health, the Medical Research Council (MRC), the National Cancer Institute of Canada (NCIC) and by Merck Research Laboratories. P.N. is supported by an MRC Postdoctoral Fellowship. Address correspondence to Dr. Frank L. Graham, Department of Biology and Pathology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1 Canada. e-mail: graham@mcmaster.ca

Received 9 March 2000; accepted 15 May 2000.

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