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# Generation of infectious genome of bovine adenovirus type 3 by homologous recombination in bacteria

Alberto L. van Olphen, Suresh K. Mittal *

Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN *47907-1243*, USA

Abstract

The widely used technique of generating adenovirus vectors by homologous recombination in mammalian cells is usually not very efficient. This communication describes a simple method of generating a plasmid containing the full-length genome of an adenovirus by homologous recombination in bacteria. Following transfection of a suitable mammalian cell line with the full-length adenovirus genome, infectious virus progeny could easily be generated. Using this technique the generation of adenovirus recombinants would be efficient and straightforward. 

Keywords: Bovine adenovirus; Adenovirus vector; Recombination in E. coli; Gene therapy

Recombinant adenoviruses are used widely as vectors to study gene expression, to transfer genes into mammalian cells and to develop recombinant vaccines (Graham and Prevec, 1992; Rosenfeld et al., 1992; Engelhardt et al., 1994; Imler, 1995; Addison et al., 1995). Foreign gene insertion in adenoviruses is usually made at two locations: the early region (E) 1 (E1) and E3 to generate recombinant adenoviruses (Berkner and Sharp, 1984; Haj-Ahmad and Graham, 1986). The basic strategy includes construction of an E1 or E3 transfer vector containing approximately 10 kb of the left- or right-end genome with an appropriate E1 or E3 deletion, respectively replaced by a foreign gene insert. Cotransfection of a suitable cell line with the transfer plasmid and the adenovirus genome or a plasmid containing almost the entire adenovirus genome eventually results in generation of an adenovirus recombinant by homologous recombination. A number of cotransfection experiments are usually needed to obtain a recombinant virus.

The recA protein of Escherichia coli (E. coli) recognizes homologous sequences between a nascent single-stranded DNA molecule and those present in double-stranded DNA and produces their recombination (Bubeck et al., 1993; Degryse, 1995). By utilizing the highly efficient homologous recombination machinery of bacteria, a desired adenovirus recombinant could easily be constructed. This strategy has been used successfully for generating human adenovirus recombinants (Chartier et al., 1996; Crouzet et al., 1997; He et al., 1998). To determine whether the same approach could be used to construct non-human adenovirus recombinants, we exploited the homologous recombination process of bacteria to generate full-length, infectious clones of bovine adenovirus type 3 (BAV3).

In order to produce the full-length genome of BAV3 in recombination-competent bacteria, a plasmid is required containing approximately 1 kb BAV3 from both ends and the BAV3 genome. The 12 kb left-end Xba I-B fragment of BAV3 (from nucleotide 1 to 12 189; for BAV3 nucleotide numbering, see Reddy et al., 1998) was cloned into the Sma I–Xba I site of an Eco RI-deleted pUC18 to generate pMvOB02. Plasmid pMvOB02 was digested with Eco RI (present at nucleotide 1156 in the BAV3 genome) and Hin cII (present in the multiple cloning site of pUC18) and a 3.8 kb fragment containing pUC18 and the left-end of BAV3 genome was gel purified. The 3.6 kb right-end Eco RI-E fragment of BAV3 genome (from nucleotide 30 833 to 34 446) was inserted into the 3.8 kb Eco RI– Hin cII fragment of pMvOB02 to obtain pMvOBE1E4. To introduce a Pac I site on either end of the BAV3 genome, the BAV3 sequences present in pMvOBE1E4 were amplified by polymerase chain reaction (PCR) using a single synthetic primer encoding the eight-nucleotide recognition sequence of Pac I followed by the first 21 nucleotides of the BAV3 inverted terminal repeat (ITR; 195 bp long ITRs are present at both ends of the

BAV3 genome). This 4.8 kb PCR product was inserted into the Pac I site of a pUC18 derivative modified by deletion of the Eco RI and Sac I sites and insertion of a Pac I linker at the Sma I site. The resultant plasmi was named pMvOBE1E4 Pac I (Fig. 1). This plasmid has a unique Eco RI site for separating the left and right ends of BAV3 sequences thereby reducing the number of background colonies containing the parental plasmid and increasing the efficiency of homologous recombination in bacteria with the BAV3 genome to generate a plasmid carrying the full-length BAV3 genome.

MDBK cells obtained from American Type Culture Collection (ATCC), were grown as monolayer cultures using Eagles minimum essential medium (MEM) (Life Technologies) supplemented with 10% fetalClone III (HyClone Laboratories) and 50 μg/ml gentamicin. BAV3 obtained from ATCC, was grown in MDBK cells, purified by cesium chloride density-gradient centrifugation and the DNA was extracted from the purified virions (Graham and Prevec, 1991).

In order to generate the complete genome of BAV3 by homologous recombination in bacteria, recA-positive E. coli strain BJ5183 (recBC, and sbcBC) (Hanahan, 1983) was cotransformed with various amounts of Eco RI-digested pMvO BE1E4 Pac 1 and uncut BAV3 DNA by electroporation (Fig. 1). Following cotransformation, ampicillin-resistant ($amp^r$) colonies were isolated, counted, grown and DNA was extracted by alkaline lysis (Sambrook, et al., 1989). To identify the clones containing the full-length BAV3 genomes, DNA was cleaved either with Bam HI, Bgl II, Eco RI, Kpn I or Xba I and analyzed on agarose gels by electrophoresis (Fig. 2). When 0.1 μg Eco RI-digested pMvOBE1E4 Pac I and 1 μg uncut BAV3 DNA was used for cotransformation, 80% of the clones were found to contain the full-length BAV3 genome (Table 1). The plasmid containing the full-length BAV3 DNA was named pMvOBAV3. All positive clones contained the full-length BAV3 genomes suggesting that there was not a major rearrangement of the BAV3 DNA in E. coli strain BJ5183. Since the amount of DNA extracted from small-scale plasmid preparations from BJ5183 was usually less compared to recombination-negative bacterial strains, the positive clones generated in BJ5183 were transferred to E. coli strain C600 or DH5 αF′ for further characterization.

The full-length BAV3 genome present in pMvOBAV3 is flanked by two unique Pac I sites (Fig. 1). Since there are no Pac I sites in the BAV3 genome, the Pac I sites present at both ends immediately before ITRs are useful in separating the BAV3 genome from pUC18 sequences. To determine whether the full-length BAV3 genome generated by homologous recombination in bacteria will retain its infectivity, transfection of a suitable mammalian cell line with clones containing the BAV3 genomes was conducted. Briefly, approximately 60% confluent MDBK cell monolayers in 60-mm dishes were transfected with four independently isolated, cesium chloride gradient-purified, Pac I-cleaved pMvOBAV3 following a liposome-mediated transfection protocol (Life Technologies). The transfected cells were incubated at 37°C for 16 h, then covered with a semi-solid agar overlay and the incubation was continued until viral plaques became visible, typically in 7–10 days. We obtained 4.5 ± 2.0 plaques/5 μg Pac I-digested pMvOBAV3. This virus was named MvOBAV3. However, more than one cotransfection experiments are usually needed to obtain a single adenovirus recombinant by homologous recombination in mammalian cells. In one such cotransfection experiment we routinely use 60 μg of adenovirus genomic DNA and 60 μg of the E1 or E3 insertion vector. Two isolated plaques from each dish were picked up, propagated in MDBK cells and virions were purified by cesium chloride density-gradient centrifugation. DNA was extracted from the purified virions and restriction patterns were compared with those obtained with BAV3 DNA (Fig. 2). Restriction patterns of MvOBAV3 with Bam HI, Bgl II, Eco RI, Kpn I or Xba I were indistinguishable from those obtained with the BAV3 genome suggesting that there was no major DNA rearrangement in the MvOBAV3 genome. All four independently isolated pMvOBAV3 produced the same results (data not shown). In addition, MvOBAV3 plaque morphology and viral titer were similar to those obtained with BAV3 (data not shown).

Using the technique of homologous recombination in mammalian cells, the construction of a BAV3 recombinant has been described (Mittal et al., 1995). The transfection efficiency of BAV3 DNA in permissive mammalian cells is approximately fivefold lower than that of human adenovirus type 5 (HAd5) (Mittal et al., unpublished data) and hence it would adversely affect the efficiency of generating BAV3-based vectors. Therefore, for BAV3 and other animal and human adenovirus genomes that have comparatively poor transfection efficiency compared to HAd5, the technique described here would be useful for generating adenovirus recombinants. Currently we are in the process of generating BAV3 recombinants having foreign gene inserted either in the E1 or E3 region by homologous recombination in bacteria.

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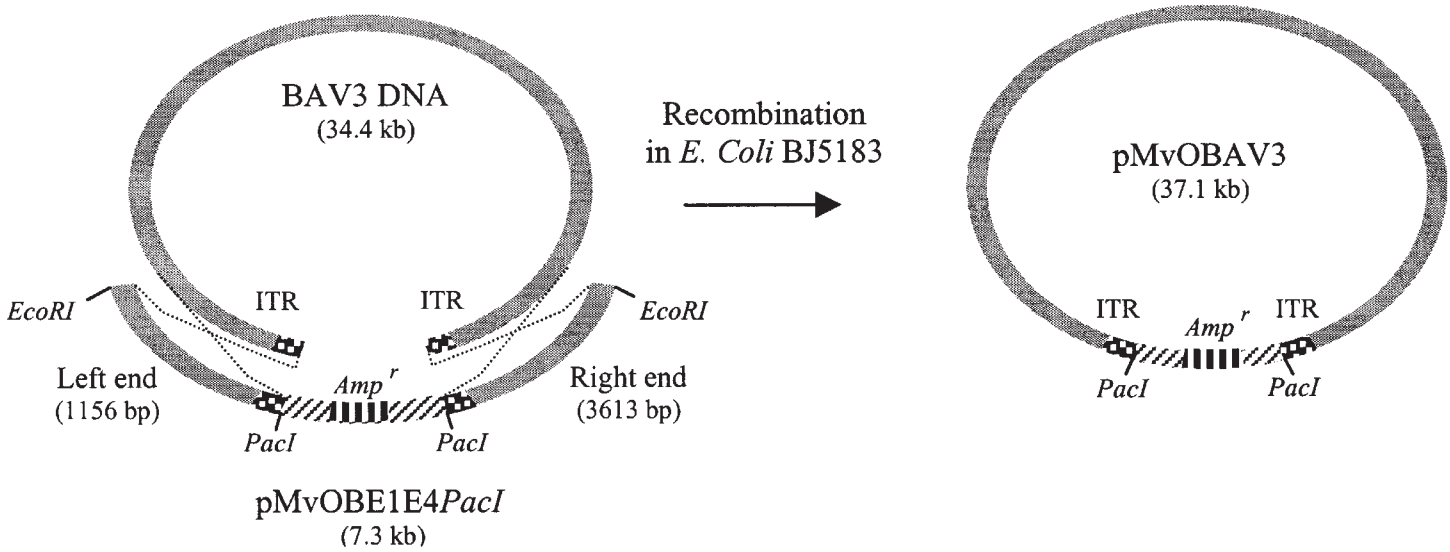


Fig. 1. Generation of the full-length BAV3 genome by homologous recombination in E. coli. BJ5183 bacteria were cotransformed with uncut BAV3 DNA obtained from purified virions and Eco RI-linearized pMvOBE1E4 Pac I. To identify the clones containing the full-length BAV3 genome, amp$^r$ bacteria colonies were grown and small-scale plasmid preparations were used for restriction pattern analyses. The plasmid containing the full-length BAV3 genome generated by homologous recombination in bacteria was named pMvOBAV3. The BAV3 sequences suitable for homologous recombination are indicated by dotted lines. The dashed lines flanking the Amp$^r$ represent the pUC18 backbone.

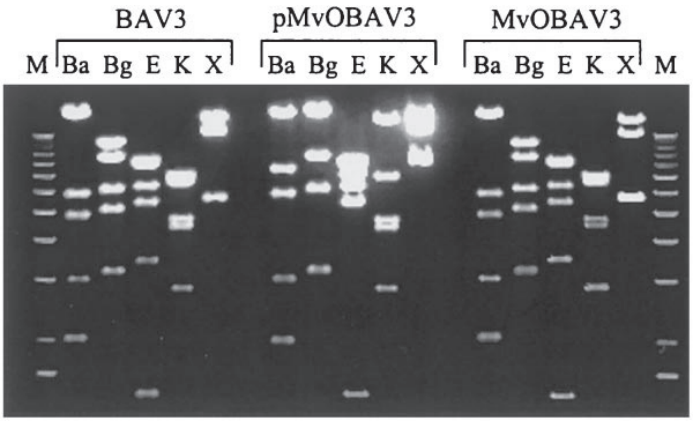


Fig. 2. Restriction endonuclease analysis of DNA obtained from BAV3, pMvOBAV3 and MvOBAV3. Purified viral (BAV3 or MvOBAV3) or plasmid (pMvOBAV3) DNA was digested with Bam HI (Ba), Bgl II (Bg), Eco RI (E), Kpn I (K), and Xba I (X) and analyzed on an agarose gel by electrophoresis. A 1-kb ladder molecular weight standard (M) was loaded in the right- and left-end lanes. The digests obtained from pMvOBAV3 yielded distinctive restriction patterns due to the fusion of one (Ba, E, K, and X) or both (Bg) terminal fragments of the BAV3 genome with the pUC18 backbone.

Table 1
Efficiency of cloning off ull-length BAV3 genomic DNA by homologous recombination in E.coli strain BJ5183

| Eco RI-digested vector pMvOBE1E4 Pac I (μg) | Insert BAV3 (μg) | Molar ratio (insert/vector) | No. of amp$^r$ colonies | Percentage of positive recombinants[a] |
|---|---|---|---|---|
| 0.01 | 0 | – | 16 | – |
| 0.01 | 0.25 | 5.3 | 19 | 0.8 |
| 0.1 | 0 | – | 13 | – |
| 0.1 | 1.0 | 2.1 | 44 | 80 |
| 1.0 | 0 | – | 9 | – |
| 1.0 | 1.0 | 0.2 | 85 | 20 |

[a] The percentage of positive recombinants is calculated by dividing the number of transformants that carry recombinant plasmids containing the full-length BAV3 genome by the total number of amp$^r$ transformants and multiplied by 100.