pAd5-Blue: Direct Ligation System for Engineering Recombinant Adenovirus Constructs

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ABSTRACT

We have engineered a new vector that makes use of direct ligation for the generation of replication-defective recombinant adenovirus constructs. In the pAd5-Blue vector, unique yet common restriction endonuclease sites exist, that allow cloning in a directional manner of a gene of interest under control of a cytomegalovirus promoter, upstream of a simian virus 40 polyadenylation signal. The insertion of the new gene replaces the β-galactosidase α gene fragment in the pAd5-Blue vector, allowing the identification of recombinants in bacterial culture by the selection of white colonies. Plasmid DNA from white colonies is digested with Pac*I and transfected into 293 cells, resulting in the generation of a homogenous population of adenovirus containing the gene of interest. The pAd5-Blue vector system does not rely on recombination either in mammalian or bacterial cells. Furthermore, because of compatible overhangs, the variety of restriction endonucleases that can be used to generate the inserted gene gives flexibility to the process for greater ease of use. The system is quick and straightforward, allowing the generation of recombinant adenoviruses within three weeks of obtaining an appropriate insert. This new vector should greatly enhance the ease and speed with which new recombinant adenovirus constructs can be made.*

INTRODUCTION

Adenovirus is a double-stranded linear DNA virus approximately 36 kb in length. Vectors constructed from adenoviruses are being used to express genes of interest for use in gene therapy and vaccine development (7,10,13,14,16). Current systems used to generate these vectors require homologous recombination in mammalian (15) or bacterial cells (2,4,9), exceedingly rare restriction endonuclease sites for direct ligation (18), Tn7 transposition elements (17), yeast artificial chromosomes (YACs) (12), or cosmids (5,6). Most of these systems make use of one adenovirus in particular, human adenovirus serotype 5 (Ad5).

Human Ad5-replication-defective constructs contain deletions in the E1 region of approximately 3000 bp, resulting in virus that can only replicate in cells that have been stably transfected with the E1 region of the adenovirus genome (i.e., 293 cells) (8). Likewise, many of these vectors contain a deletion in the E3 region of approximately 2700 bp, which results in a loss of inhibition of the major histocompatibility complex (MHC) class I response, leading to an increase in the ability of animals infected by these viruses to develop an immune response to the expressed foreign genes (3). These deletions added to the estimated 105% of genome size that adenovirus can package (1800 bp), giving vectors the ability to contain about 7500 bp of foreign sequence.

We have developed a vector, pAd5-Blue, that makes use of two unique yet common restriction endonuclease sites, *Cla*I and *Xba*I, placed in the E1-deleted region of a replication-defective, E1/E3-deleted Ad5-containing plasmid, allowing for direct ligation of genes of interest. Between these two unique restriction endonuclease sites, the plasmid contains a gene for ampicillin (Ap) resistance and a functional β-galactosidase α gene fragment (*lacZ*) that gives blue colony color in the presence of X-gal. Genes of interest can be directly ligated into pAd5-Blue between *Cla*I and *Xba*I, downstream of a cytomegalovirus (CMV) promoter, and upstream of a simian virus 40 (SV40) polyadenylation signal in a directional manner. The cloning of the gene of interest replaces the *lacZ* gene, resulting in the production of recombinants that can be selected on the basis of their white colony color. This system has flexibility in the ease of gene insertion because both *Cla*I and *Xba*I share overhang motifs with multiple other restriction endonucleases. DNA from white recombinant colonies can be easily evaluated by restriction endonuclease digestion, and the presence of the desired insert can be easily validated. Recombinant adenovirus is generated by

transfection of linearized DNA into 293 cells, with no need for subsequent plaque purification. This paper demonstrates the flexibility and ease of use of the pAd5-Blue system for the insertion and expression of desired genes in recombinant adenovirus.

MATERIALS AND METHODS

Bacterial Strains and Mammalian Cell Lines

The adenovirus plasmids and pAd5-Blue were propagated in electrocompetent cells (One Shot® TOP10® Electrocomp™ Cells; Invitrogen, Carlsbad, CA, USA). Other vectors were grown in chemically competent TOP10 cells (Invitrogen). Before designing the pAd5-Blue vector, a precursor vector containing foreign genes had been constructed using homologous recombination in competent BJ5183 cells, following the manufacturer's protocols (QBiogene, Montreal, QC, Canada). Recombinant adenoviruses derived from pAd5-Blue vectors were grown in and purified from 293 cells (8).

Construction of pAd5-Blue Plasmid

The precursor plasmid to pAd5-Blue was made in our laboratory to study the utility of an adenovirus vector containing the foot-and-mouth disease virus (FMDV) capsid and 3C proteinase coding regions as a vaccine candidate (pAd5-FMDVA24). This plasmid was constructed using AdEasy™ plasmids, cells, and protocols (QBiogene) and contained not only a unique *Xba*I site at the carboxy terminus used to clone the FMDV genes but also a new, unique *Cla*I site downstream of the *Bgl*II site used for cloning at the amino terminus (Figure 1A). The FMDV sequences were removed by digestion with *Cla*I and *Xba*I, and the vector was treated with shrimp alkaline phosphatase.

To clone the *lacZ* and Ap resistance genes into *Cla*I- and *Xba*I-digested pAd5, an intermediate plasmid was constructed. The *lacZ* and Ap genes were removed from Litmus 38 (New England Biolabs, Beverly, MA, USA) by digestion with *Swa*I, which leaves a blunt end, and BspLU11I, which was filled in. The blunt-ended *lacZ*- and Ap-containing fragment was ligated into *Eco*RV-digested pBluescript® II KS$^+$ (Stratagene, La Jolla, CA, USA) between unique *Cla*I and *Xba*I sites (Figure 1B, pKS-lacZAp). The *lacZ* and Ap genes, which do not contain *Cla*I or *Xba*I sites, were removed from pKS-lacZAp by digestion with *Cla*I and *Xba*I and ligated into *Cla*I/*Xba*I-digested and dephosphorylated pAd5 to form pAd5-Blue. pAd5-Blue retains the unique *Cla*I and *Xba*I sites for cloning genes between the CMV promoter and SV40 polyadenylation signals (Figure 1C, pA).

In the presence of X-gal and the antibiotics Kn and Ap, pAd5-Blue yields blue colonies that can be selected, and large quantities of DNA are produced. This DNA was digested individually with *Xba*I and *Cla*I, ethanol precipitated, incubated with shrimp alkaline phosphatase, ethanol precipitated, and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. DNA prepared in this manner is stable for over three months at 4°C.

Construction of pAd5-Blue Containing Genes of Interest

Digestion of the gene of interest with *Cla*I or *Cla*I-compatible restriction endonucleases and *Xba*I or *Xba*I-compatible restriction endonucleases allows for insertion of this gene between the *Cla*I and *Xba*I sites of pAd5-Blue in place of the *lacZ* and Ap genes. The chimeric DNA is transformed into electrocompetent TOP10 cells for efficient insertion into bacteria. Because this protocol does not require homologous recombination in recombination-competent cells, there is no need for concern about random recombination events interfering with the generation of the desired product. Desired clones

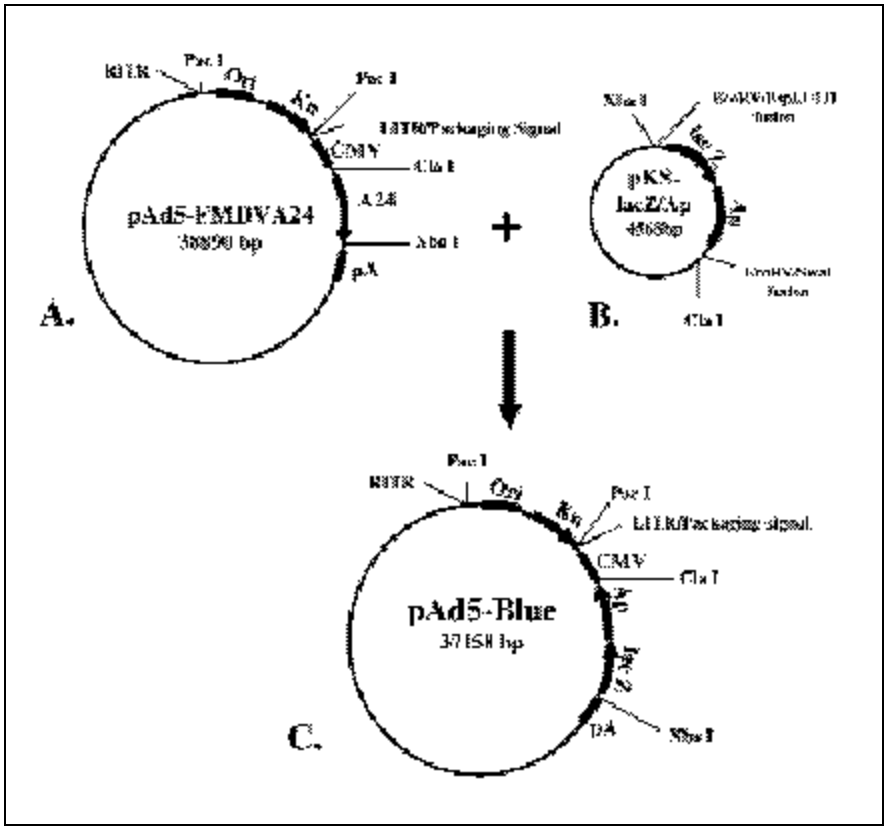

Figure 1. Construction of pAd5-Blue. pAd5-FMDVA24 DNA was digested with *Cla*I and *Xba*I to remove the FMDV A24 sequence and was ligated to the Ap and *lacZ* genes from *Cla*I- and *Xba*I-digested pKS-lacZAp to form pAd5-Blue. pAd5-Blue contains the *lacZ* gene and Ap and Kn resistance genes. In the presence of X-gal and the above antibiotics, pAd5-Blue colonies are blue and can be easily selected. Ori is a bacterial origin of replication, RITR and LITR are the right and left inverted terminal repeat regions of Ad5, respectively, and CMV and pA represent the promoter and SV40 polyadenylation signal, respectively.

develop as white colonies in the presence of X-gal and can be separated from the few pAd5-Blue uncut background colonies. To demonstrate that selected white colonies contain the gene of interest, DNA can be digested with restriction endonucleases such as *Hin*dIII and directly compared to similarly digested pAd5-Blue. At this step, a given clone of proper size represents a single population of adenovirus DNA.

Generation of Recombinant Virus in 293 Cells

*Pac*I-linearized pAd5-Blue-recombinant plasmid DNA (3–3.5 µg) was transfected into a well of a 6-well plate containing 293 cells (Transfection MBS Mammalian Transfection Kit; Stratagene), following the manufacturer's instructions. Wells were examined daily after day 3, and plaques were picked as they appeared, usually 4–8 days after transfection. The plaques were individually used to infect a 150-mm flask of 293 cells when it was approximately 60% confluent. Upon the development of cytopathic effects, usually 2–3 days after infection, the cells were centrifuged at 1500–2000× *g* for 10 min and resuspended in PBS containing calcium and magnesium (PBS^{++}) and Nonidet™ P40 detergent, added to a concentration of 0.5%. The cells were frozen and thawed five times, and cellular debris was pelleted by centrifugation at maximum speed in a model 5417C microcentrifuge (Eppendorf Scientific, Wesbury, NY, USA) for 5 min. The supernatant was removed and centrifuged as above. The resulting supernatant represented a high-titer crude stock of virus, and viral DNA was extracted and examined by *Hin*dIII digestion for the appropriate gene fragments. This virus stock was used to infect multiple 150-mm flasks of 293 cells for large-scale preparation of virus and subsequent purification by discontinuous, followed by continuous, CsCl gradient centrifugation.

RESULTS AND DISCUSSION

Generation and Purification of pAd5-Blue

The ligation of the XbaI-lacZ-Ap-ClaI fragment from the pKS-lacZAp plasmid, with the pAd5-FMDVA24 plasmid from which the FMDV A24 sequences had been removed, resulted in 267 colonies on Kn/Ap plates after electroporation into electrocompetent TOP10 cells, indicative of the presence of the Ap resistance gene in the insert. Of these colonies, eight were picked and grown in broth containing Kn/Ap and streaked onto Kn/Ap plates containing X-gal. All eight clones yielded blue colonies on Kn/Ap/X-gal plates, indi-

Table 1. *Cla*I- and *Xba*I-Compatible Restriction Endonucleases

*Cla*I-Compatible Enzymes		*Xba*I-Compatible Enzymes	
Enzyme	**Recognition Sequence**	**Enzyme**	**Recognition Sequence**
*Cla*I	AT! CGAT	*Xba*I	T! CTAGA
*Acl*I	AA! CGT T	*Avr*II	C! CTAGG
*Bst*BI	TT! CGA A	*Nhe*I	G! CTAGC
*Nar*I	GG! CGC C	*Spe*I	A! CTAGT
*Acc*I[a]	GT! CGA C		
*Bsa*HI	G R[b]! CGY[c] C		
*Hin*P1I	G! CGC		
*Hpa*II	C! CGG		
*Taq*I	T! CGA		

[a]*Acc*I also recognizes the sequence GTATAC, which is not *Cla*I-compatible.
[b]R represents a purine.
[c]Y represents a pyrimidine.
Underlined bases indicate the overhang after restriction endonuclease digestion.
! represents the cut site of each restriction endonuclease.

cating the presence of the *lacZ* gene. DNA prepared from all eight colonies had banding patterns on agarose gels after digestion with *Bam*HI, *Ssp*I, and *Cla*I/*Xba*I, which was indicative of the insertion of the lacZ-Ap fragment into an otherwise full-length, replication-defective Ad5 background. One colony was chosen as representative, and a large-scale CsCl preparation of DNA was made (1).

Generation of pAd5 Constructs: Example of the Use of pAd5-Blue to Form Recombinant Ad5 Virus

In pAd5-Blue, the *Cla*I site is upstream from the translation start codon of an inserted gene, and the *Xba*I site is downstream from the inserted gene's stop codon. The use of unique yet common restriction endonucleases imparts a level of flexibility to gene insertion in this system that is not seen in any other adenovirus system currently available. As shown in Table 1, there are eight other restriction endonucleases in addition to *Cla*I that share the same two base pair 5′ overhangs as *Cla*I and can thus be used for ligation into this site. There are three other restriction endonucleases besides *Xba*I that share the four base pair *Xba*I 5′ overhang and are thus potentially useful for ligation into this site. In addition to this flexibility, ligation is directional in nature, assuring the insertion of genes in only one possible orientation.

Genes of interest lacking *Cla*I and *Xba*I sites can be PCR amplified to contain these sites at their 5′ and 3′ termini, respectively, and directly ligated into *Cla*I/*Xba*I-digested pAd5-Blue, or they can be cloned into an appropriate shuttle vector containing *Cla*I and *Xba*I sites. Genes of interest containing internal *Cla*I or *Xba*I sites can be modified by PCR to contain *Cla*I- and *Xba*I-compatible sites not found internally in the gene.

We have constructed at least 11 recombinant viruses using the pAd5-Blue system, with insert sizes ranging from 511 to 6431 bp. The genes of interest were either PCR amplified (porcine interleukin 4 and porcine gamma interferon, etc.) or removed from vectors (vesicular stomatitis G protein gene and portions of the FMDV genome) and cloned into pAd5-Blue. Generally, we found that after transformation, most of the colonies are white, and 80%–100% of the white colonies have the correct band pattern after restriction endonuclease digestion. Purified virus obtained from transfection of linearized recombinant pAd5-Blue into 293 cells,

followed by amplification and purification, are routinely on the order of 10^{10} pfu/mL. Ad5 recombinant viruses demonstrated foreign gene expression in 293 cells or cells nonpermissive for adenovirus replication as assayed by radioimmunoprecipitation, immunoflurorescence, or western blotting and induced antibody production in mice and swine (data not shown).

Utility of the pAd5-Blue System

This system combines the ease of direct insertional cloning into unique restriction endonuclease sites with the flexibility of ligation to multiple sites that share compatible overhangs. The selection of colonies by white color simplifies the process of identifying recombinant clones. The requirement for recombination to generate chimeric viral DNA and the need for plaque purification are eliminated because each plaque derived from a linearized plasmid clone represents an individual population by definition and allows for the generation of recombinant viruses within three weeks. Furthermore, the use of standard recombinant DNA methods to generate chimeric Ad5 viruses, with no need for expertise in specialized techniques such as Tn7 transposition elements (17), YACs (12), or cosmids (5,6), should make this system attractive to many laboratories.

The pAd5-Blue system has become an important tool for the expression and examination of biological activity of various genes in our laboratory and in other laboratories at our institute. We are currently examining the utility of these virus vectors in animals as tools for disease control.

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Electrophoretic Mobility Shift Scanning Using an Automated Infrared DNA Sequencer

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ABSTRACT

Electrophoretic mobility shift assay (EMSA) is widely used in the study of sequence-specific DNA-binding proteins, including transcription factors and mismatch binding proteins. We have established a nonradioisotope-based protocol for EMSA that features an automated DNA sequencer with an infrared fluorescent dye (IRDye™) detection unit. Our modification of the elec-