JOURNAL OF VIROLOGY, Mar. 1997, p. 1842–1849 Vol. 71, No. 3
0022-538X/97/$04.00+0

Construction of Adenovirus Vectors through Cre-*lox* Recombination

STEPHEN HARDY,* MARINA KITAMURA, TONJA HARRIS-STANSIL, YUMIN DAI, AND M. LISA PHIPPS

Somatix Therapy Corporation, Alameda, California 94501-1034

Received 9 September 1996/Accepted 15 November 1996

Two barriers prevent adenovirus-based vectors from having wide application. One is the difficulty of making new adenoviruses, and the second is the strong immunological reaction to viral proteins. Here we describe uses of Cre-*lox* recombination to overcome these problems. First, we demonstrate a simple method for constructing E1-substituted adenoviruses. Second, we demonstrate a method to construct adenovirus vectors carrying recombinant genes in place of all of the viral genes, so-called gutless adenovirus vectors. The pivotal feature in each method is the use of a negatively selected adenovirus named Ψ5. We engineered a *cis*-acting selection into Ψ5 by flanking its packaging site with *loxP* sites. When Ψ5 was grown in cells making a high level of Cre recombinase, the packaging site was deleted by recombination and the yield of Ψ5 was reduced to 5% of the wild-type level. To make a new E1-substituted virus, we used Ψ5 as a donor virus and recombined it with a shuttle vector via a *loxP* site. The resulting recombinant virus has a single *loxP* site next to the packaging site and therefore outgrows Ψ5 in the presence of Cre recombinase. To make a gutless virus, we used Ψ5 as a helper virus. The only viral sequences included in the gutless vector are those needed in *cis* for its replication and packaging. We found that a *loxP* site next to the packaging site of the gutless virus was necessary to neutralize homologous recombination between Ψ5 and the gutless viruses within their packaging domains.

Adenoviruses are attractive candidates for many gene delivery applications in medicine and science. Two particular features of adenovirus biology could be critical to successful use in gene therapy or molecular genetic experiments. First, the virus infects both resting and dividing cells of many types. Second, highly purified solutions of virus can easily be produced with titers of up to 10^{13} particles/ml. Additionally, decades of research have produced a relatively clear picture of the viral life cycle and most functions of the many viral proteins (reviewed in reference 24). Despite these attractive features, adenovirus has been only narrowly applied as a tool. A major barrier to general application of the virus is the process of making recombinant viruses. Generally, new adenoviruses are constructed by a process of overlap recombination followed by screening and plaque purification (3, 6, 20, 25). These steps are inefficient, technically demanding, and very time-consuming.

For most in vivo applications, there is a second serious limitation of adenoviruses. Adenoviruses are highly immunogenic, a property which has been maintained in current adenovirus vectors. The immunological reaction against adenoviruses can be thought of as having two phases, an initial reaction to the capsid proteins of the infecting virus and a later reaction against cells synthesizing viral proteins. Because of the complexity of adenovirus, most of the genome has been left intact in current vectors, and the remaining viral genes are able to direct protein synthesis, albeit at a reduced level. Therefore, cells transduced by these vectors are eliminated when viral genes in the vector begin to synthesize proteins (8, 28). Thus, these E1-deleted adenovirus vectors are powerful tools for experimentation in tissue culture systems and are restricted in their therapeutic uses to applications where only transient gene expression is needed or to those where a stimulation of immunity is beneficial. The most practical route to overcome the immunological barrier to long-term gene transfer is to remove all of the viral genes from the vector, producing a "gutless" virus.

Keeping these difficulties in mind, we have developed methods for creating recombinant adenoviruses by using Cre recombinase from phage P1. Recombinant virus construction is driven by selection against a special adenovirus virus which we call Ψ5. The negative selection is based on the observation that deletion of a sequence from 194 to 358 bp in the left end of adenovirus prevents the viral chromosome from being packaged into capsids (10, 11, 15). To make a conditional version of such a deletion, we flanked the packaging site in Ψ5 by directly repeated *loxP* sites and constructed a 293 cell line called CRE8 which makes a high concentration of Cre recombinase (23). In these cells, recombination will delete the intervening packaging sequence in Ψ5, producing an unpackageable chromosome.

Using Ψ5 and CRE8 cells, we have developed two methods to make either E1-substituted or gutless adenovirus vectors. To make an E1-substituted virus, we used Ψ5 as a donor virus to supply the viral backbone. In addition to supplying negative selective pressure, Cre recombinase can catalyze recombination between Ψ5 and a shuttle plasmid with a single *loxP* site, providing an efficient means to construct recombinants. Selective growth of the recombinant viruses was ensured by installing a normal packaging sequence on the shuttle plasmid. This combination of selection and recombination provided by Cre protein set up an arrangement to rapidly create new viruses.

In our second method, we used Ψ5 as a helper virus to support replication and packaging of a gutless virus. Such a virus contains only those terminal sequences needed for replication and packaging of the viral chromosome surrounding nonviral DNA. The inverted terminal repeats (ITR) which serve as the origins of replications and a packaging sequence are two such sequences. Since the packaging site in the gutless virus does not have flanking *loxP* sites, the gutless virus should

* Corresponding author. Mailing address: Somatix Therapy Corporation, 850 Marina Village Pkwy., Alameda, CA 94501-1034. Phone: (510) 748-3029. Fax: (510) 769-8533. E-mail: shardy@apple.somatix.com.

be preferentially packaged. Growth of a mixed population of helper and gutless viruses on CRE8 cells should amplify the gutless virus.

MATERIALS AND METHODS

Cells and viruses. 293 and CRE8 cells were cultured in Dulbecco modified Eagle medium with 10% calf serum (12). The selective cell line CRE8 has a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. The construction and properties of CRE8 cells are described elsewhere (13a). Viruses were used as freeze-thaw lysates, and all infections were done at a multiplicity of infection of 5 for each virus. Transfections were done according to Graham and van der Eb (13). Typically, a confluent 10-cm-diameter dish of 293 or CRE8 cells (1.6×10^7) was split into 5- to 6-cm-diameter dishes for transfection 2 to 4 h later. Each dish received 3 μg of pAdlox or ploxAβ plasmid and 3 μg of Ψ5 viral DNA in a final volume of 0.5 ml of $CaPO_4$, which was applied to the cells for 16 h. For the transfections using ploxAβ shuttle plasmid, the medium was removed after 3 days and 1% Noble agar overlay in Dulbecco modified Eagle medium with calf serum was added. At 7 days, another layer of agar overlay containing 0.8 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml was added. Blue plaques were removed 1 day later.

For the experiment to determine the optimal way to deliver the Ψ5 donor DNA, three plates of CRE8 cells were transfected with *Sfi*I-digested pAdlox β-gal DNA and infected with Ψ5 virus at three different times, 4 h before transfection, at the same time as transfection, and at 18 h after transfection. Lysates were made 2 days after infection. The virus was then passed sequentially through CRE8 cells twice, and the packaged DNA was prepared from the second passage. For comparison, cells were cotransfected with Ψ5 and pAdlox β-gal DNAs. After 8 days, lysate from the cotransfection was used to infect CRE8 cells for packaged DNA. Finally, the DNA was digested with *Bsa*BI to distinguish between Ψ5 and the recombinant DNAs.

Construction of vectors. Standard techniques were used to construct all plasmids. Plasmids were purified by the polyethylene glycol-LiCl technique (2). Frequently, rapid-prep DNA prepared by the alkaline lysis protocol was used for transfection to construct E1-substituted viruses (2). The success rate was similar to that for assays using purified plasmids (data not shown).

pCMV Ad, pAdlox, pAβ, and derivatives. The shuttle plasmids for either E1-substituted or gutless virus construction were based on adenovirus type 5 (Ad5) and are derivatives of pCMV Ad. Each contains the following sequences in this order: (i) a left ITR, (ii) a packaging sequence, (iii) an expression cassette, and (iv) a right ITR (Fig. 1). Specifically, pCMV Ad begins with an *Sfi*I site, GGGCCGCTGCGGCC, followed by nucleotides 2 to 553 from Ad5 (nucleotide 1 is the last C of the *Sfi*I site). This is connected to an *Xho*I site followed by the human cytomegalovirus immediate-early promoter from −600 to +1 relative to the start of transcription. The next segment is the polylinker of pSP73 from *Hin*dIII to *Eco*RI (Promega) and then polyadenylation signals from simian virus 40 (nucleotides 2752 to 2534 in the simian virus 40 genome). The poly(A) signals are followed by a pSP73 fragment from nucleotides 22 back through 2382 (containing *Cla*I, *Eco*RV, and *Bgl*II sites and an SP6 promoter). An *Apa*I site then connects this sequence to the right ITR, which ends with another *Sfi*I site. The *Sfi*I-bounded fragment is cloned between the *Pvu*II sites of pBluescript$^+$, destroying the *Pvu*II sites (bases 533 back to 979) (Stratagene). Digestion of pCMV Ad with *Sfi*I cleaves the plasmid sequence away from the Ad5-based portion, allowing the Ad5 sequences to replicate in adenovirus-infected cells (data not shown) (14). To create pAdlox, a *loxP* site was inserted between the *Cla*I and *Bgl*II sites in pCMV Ad, making pAdlox. The new sequence between *Cla*I and *Bgl*II is ATCGATCCATAACTTCGTATAATGTATGCTATACGAAGTTAT CCAGATCT. (Note that the *Cla*I site will be methylated if the plasmid is grown in *dam*$^+$ *Escherichia coli*.)

To monitor either overlap recombination or Cre-*lox*-mediated recombination, β-galactosidase reporter genes were inserted between the *Hin*dIII and *Sma*I sites of pAdlox or pCMV Ad. The *Bgl*II B fragment of Ad5 (3328 to 8914) was inserted into the unique *Bgl*II site in pCMV Ad, making pCMV Ad B with a β-galactosidase reporter gene. The shuttle plasmids were cut with *Sfi*I where indicated in Table 1.

The gutless shuttle plasmid pAβ was constructed from pCMV Ad. The *Bgl*IIA fragment from λ phage (nucleotides 22425 back to 415 in λ) was inserted between the *Bam*HI and *Bgl*II sites of pCMV Ad. Then a 4.9-kb β-galactosidase expression cassette was removed from pON249 (a gift of J. Michael Bishop) by digestion with *Bam*HI and ligated into the *Bgl*II site, creating pAβ. The size of the resulting ITR-bounded fragment is 28,350 bases. The gutless plasmid ploxAβ was made from pflox Pac (see below) and is identical to pAβ, except that it has a single *loxP* site at position 193 relative to the left adenovirus origin.

Ψ5 and Adβ-gal viruses. The Ψ5 virus is an E1- and E3-deleted version of Ad5 containing *loxP* sites flanking the packaging site (Fig. 1). Ψ5 was constructed by overlap recombination between a modified version of pAdlox and Adβ-gal (8, 25). A second *loxP* site was inserted between nucleotides 193 and 194, the Ad5 left-end sequence in pAdlox, creating pflox Pac. The orientations of the *loxP* sites are the same, and recombination between them will remove the packaging site. Then the *Bgl*II B fragment of Ad5 was inserted into the *Bgl*II site of pflox Pac to create pflox PacB. *Sfi*I-digested pflox PacB plasmid DNA and *Cla*I-digested Adβ-gal viral DNA were introduced into 293 cells by using $CaPO_4$ transfection. Ψ5 was isolated by plaque purification using standard techniques. Adβ-gal is an Ad5 recombinant made from a pCMV Ad B plasmid containing a β-galactosidase gene and *dl*324 (27). The expression cassette and β-galactosidase gene replace E1 sequences up to nucleotide 3328, and a deletion of nucleotides 28592 to 30470 is in the E3 region.

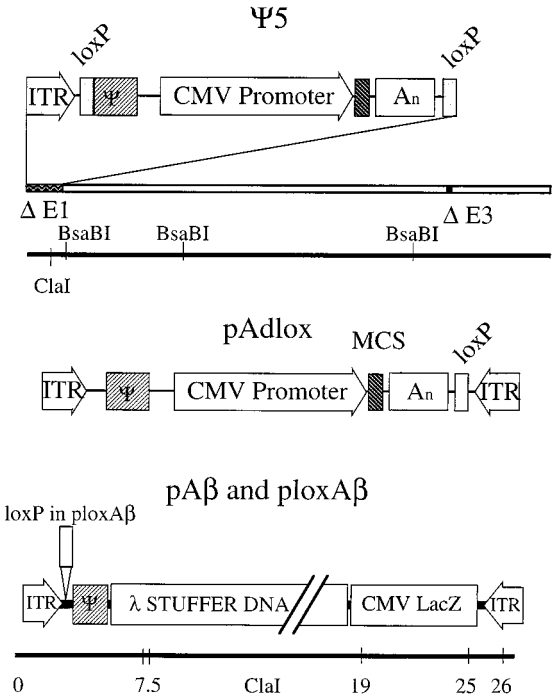

FIG. 1. Vector maps. Ψ, packaging site; CMV, cytomegalovirus; A_n, polyadenylation signal; MCS, multiple cloning site. (Top) A map of Ψ5 virus. The *loxP* sites are directly repeated. The single *Cla*I site is at 1473 between A_n and the right *loxP* site. The three *Bsa*BI sites are at 2249, 9972, and 21620. (Middle) A map of the pAdlox shuttle plasmid for making E1-substituted adenoviruses. DNA sequences inserted between the Ψ site and the *loxP* site will be incorporated into recombinant viruses in a Cre recombinase-catalyzed recombination between pAdlox and Ψ5. (Bottom) A map of shuttle vectors for making gutless adenoviruses. pAβ and ploxAβ are identical except that ploxAβ has a *loxP* site inserted at position 194 between the left ITR and the packaging site. The positions of *Cla*I sites are marked.

Viral DNA analysis. Total low-molecular-weight DNA was prepared as described by Hirt (16). Packaged viral DNA was prepared by using spermine precipitation to remove the unpackaged nucleic acids as follows. At 48 h after infection, a 10-cm-diameter dish of infected cells was harvested by suspending the cells in their media and centrifuging them in a clinical centrifuge. The pellet was suspended in 400 μl of 10 mM Tris (pH 9)–1 mM EDTA; then 400 μl of 20% ethanol–100 mM Tris (pH 9)–0.4% sodium deoxycholate was added, and this suspension was passed through a pipette tip 15 times. The unpackaged nucleic acids were removed by adding 8 μl of 500 mM spermine-HCl, mixing, incubating the mixture for 10 min on ice, and centrifuging it in a microcentrifuge at full speed for 4 min at 4°C. The supernatant was digested with 4 μl of RNase A (10 mg/ml) for 10 min at 37°C. Next, 60 μl of 10% sodium dodecyl sulfate, 20 μl of 0.5 M EDTA, and 40 μl of pronase (50 mg/ml) were added, and the sample was incubated 1 h at 40°C. This sample was extracted once with phenol-chloroform and then precipitated with isopropanol. The DNA was dried and suspended in 25 μl of 10 mM Tris (pH 8)–1 mM EDTA, and a 4-μl aliquot was digested with the appropriate enzyme. A 1-kb DNA ladder (Life Technologies) was run on all agarose gels for size standards. DNA fragments were resolved by electrophoresis through 1% agarose gels run in Tris-acetate-EDTA, visualized with ethidium bromide, and photographed. Band intensities were determined from photographic negatives, using a laser densitometer (Molecular Dynamics). The relative molar yield of each left-end fragment shown in Fig. 2a was calculated by correcting for background fluorescence, scaling for yield by using the 2.4-kb Adβ-gal fragment, and then adjusting for the number of base pairs in each fragment.

Cell staining and flow cytometry. To determine relative amounts of recombinant β-galactosidase-expressing virus, 1 ml of lysate was used to infect a 6-cm-diameter dish of 293 cells. At 20 to 24 h postinfection, the cells were removed with trypsin and aliquots were loaded with fluorescein-digalactoside (FDG) for

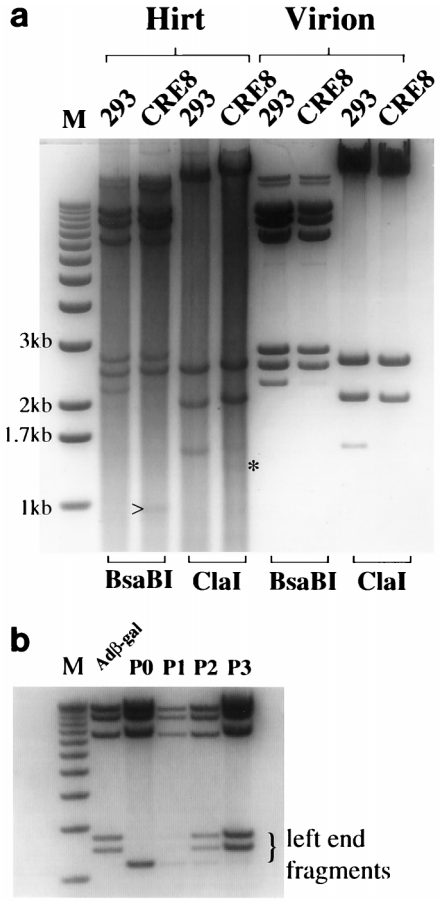

FIG. 2. (a) Restriction mapping of Adβ-gal and Ψ5 DNA coinfected into 293 or CRE8 cells. For each cell type, total low-molecular-weight (Hirt) DNA or packaged DNA was prepared and digested with *Bsa*BI or *Cla*I and resolved by agarose gel electrophoresis. The viruses are identical except in their left ends. Digestion of Adβ-gal with *Bsa*BI produces 2.4- and 2.8-kb fragments, and digestion with *Cla*I produces 2.1- and 2.5-kb bands in either cell type. *Cla*I cleaves between the *loxP* sites in Ψ5, producing a 1.5-kb fragment from unrecombined Ψ5 DNA. *Cla*I does not cleave the recombined form of Ψ5 but does produce a 1.3-kb fragment from the excised circle marked with an asterisk. *Bsa*BI cuts after the *loxP* sites and produces fragments of 2.2 kb from unrecombined Ψ5 DNA and 0.98 kb (marked with >) from recombined Ψ5 DNA. The lane M contains a 1-kb DNA ladder. (b) Negative selection against Ψ5 virus in CRE8 cells. Lane M contains a 1-kb DNA ladder for size standards. The next lane is a digest of Adβ-gal DNA. Lane P0 is a reconstruction of 1% Adβ-gal and 99% Ψ5 DNAs mixed and digested. Lanes P1, P2, and P3 are packaged DNAs prepared from three successive infections of mixed viruses containing 1% Adβ-gal and 99% Ψ5 on CRE8 cells. All viral DNAs were digested with *Bsa*BI, and the virus-specific left-end fragments are bracketed in the right margin.

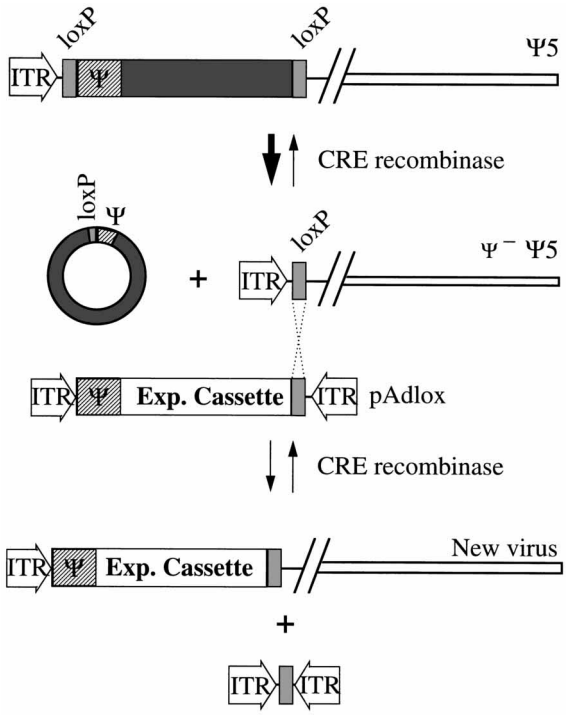

FIG. 3. Construction of an E1-substituted adenovirus by using Ψ5 and a shuttle vector. Negative pressure on Ψ5 is achieved by intramolecular recombination removing the packaging site in the first step. An intermolecular recombination between the shuttle vector and Ψ5 then creates a new virus which has an intact packaging site and carries a recombinant gene, marked Exp. Cassette. The packaging site is labeled Ψ.

60 s (22). Cells were stained with propidium iodide and analyzed with a Becton Dickinson FACScan. Data were collected from viable single cells.

Nucleotide sequence accession number. The GenBank accession number for pAdlox is U62024.

RESULTS

Selection against Ψ5 virus. As noted above, the Ψ5 virus is designed so that it can be selected against. When Ψ5 is propagated in the presence of Cre recombinase, its *cis*-acting packaging sequence, which is flanked by *loxP* sites, is deleted, and the resulting genome cannot be encapsidated. To understand the selection process in detail, we examined Ψ5 virus grown in nonselective (293) or selective (CRE8) cells. As a standard, we used a virus with a normal packaging sequence (Adβ-gal) in coinfections with Ψ5. These two viruses are identical except for their left ends, which are easily distinguished by restriction analysis of the viral DNAs. Comparison of either total low-molecular-weight DNA (Hirt extraction) or packaged DNA provides the means to judge the relative packaging and replication efficiencies and to identify the recombination products.

Digestion of Ψ5 DNA with *Bsa*BI should produce a 2.2-kb fragment before recombination and a 0.98-kb fragment after recombination (*Bsa*BI digestion of Adβ-gal produces 2.4- and 2.8-kb fragments). In the Hirt-extracted DNAs, the predicted 0.98-kb band appeared in CRE8 cells but not in 293 cells (Fig. 2a). The degree of recombination was over 90%, as judged by the relative intensity of the 2.2- and 0.98-kb bands. The amount of total Ψ5 left ends in 293 or CRE8 cells relative to the amount of Adβ-gal left ends was constant. In the *Cla*I digest of Hirt DNAs, the 1.5-kb Ψ5 left-end fragment was greatly reduced in CRE8 cells and there was a faint fragment of 1.3 kb, the predicted size of the excised circle (ClaI digestion of Adβ-gal yields 2- and 2.4-kb fragments).

If the packaging site was removed from Ψ5 in CRE8 cells, then Ψ5 DNA should be underrepresented in the packaged DNA from these cells. This was in fact the case. In 293 cells, the yields of the two DNAs were about the same. However, for growth in CRE8 cells, the Ψ5 left-end fragment was about 10% of the Adβ-gal fragments when the DNA was digested with either enzyme. Of the Ψ5 DNA which was packaged, the *Bsa*BI digestion shows that it was unrecombined, since the 2.2-kb band was present and the 0.98-kb band was absent from the packaged DNA. These data show that Ψ5 virus was subjected to a strong negative selection in CRE8 cells.

To confirm that the Adβ-gal virus rapidly overgrows the negatively selected Ψ5 virus in CRE8 cells, we mixed it with Ψ5

TABLE 1. Comparisons of Cre-*lox*- and homology-driven recombination and of viral and plasmid DNAs as sources of the virus backbone

Shuttle vector	Mode of recombination	Type of donor DNA		% *lacZ*-positive cells in FDG assay			
		Viral	Plasmid	3 days	7 days	10 days	14 days
pAdlox, cut	CRE-*lox*	Ψ5		1.7	100		
pAdCMV B, cut	Homology	Ψ5		0.2	100		
	Homology		pBHG10	0	0	0.65	100
pAdCMV B	Homology		pBHG10	0	0	0	0

at a ratio of 1 to 100 and infected CRE8 cells serially three times. At each passage, we prepared packaged DNA and monitored the ratios of the two viruses by restriction analysis. Figure 2b shows that in the course of three passages, the Ψ5-specific fragment all but disappeared and the Adβ-gal fragments became predominant.

E1-substituted virus construction. By using selection against Ψ5, it should be possible to make a recombinant adenovirus carrying nonviral DNA in place of the E1 genes by cotransfecting a shuttle vector with a *loxP* site (pAdlox) and Ψ5 DNA into a CRE8 cells (Fig. 3). In the first step of the reaction, Cre recombinase should catalyze recombination between the two *loxP* sites in Ψ5, removing the packaging site from the virus. In the second step, Cre recombinase should catalyze a recombination between Ψ5 and pAdlox, transferring the recombinant genes into Ψ5. The resulting recombinant virus will now have a single *loxP* site and therefore will have a considerable growth advantage over Ψ5 in CRE8 cells. This growth advantage should generate virus stocks comprised predominantly of the recombinant virus.

We compared our Cre-*lox* recombination technique to a popular overlap recombination method for production of recombinants (3). We set up a series of recombinations between transfected adenovirus donor DNAs and shuttle vectors and then prepared lysates 3, 7, 10, and 14 days after transfection and screened for the presence of *lacZ*-positive virus, using the FDG assay (Table 1; see Materials and Methods). These recombinations were used to examine the effects of two factors, the source of the donor DNA and the mechanism of recombination. First we transfected either Ψ5 viral DNA or pBHG10 (3) plasmid DNA as the donor genome along with shuttle vectors marked with *lacZ* genes. Second, we combined Ψ5 donor DNA with *lacZ*-marked shuttle vectors having either a *loxP* site for Cre-*lox* recombination or a 5.5-kb adenovirus fragment for overlap recombination. Recombination into Ψ5 viral DNA by either method produced a *lacZ* virus by 4 days, with Cre-*lox* recombination being slightly more efficient. In contrast, overlap recombination into pBHG10 plasmid DNA required 10 days before *lacZ* virus appeared, and then it did so only when the linear shuttle vector was used.

An important factor in the recombination process might be the topology of the shuttle plasmid. To test this, we transfected pAdlox plasmids carrying a β-galactosidase marker gene (pAd lox β-gal) either uncut or treated in various ways along with Ψ5 viral DNA (Fig. 4a). After 3 days, we prepared lysates and titered them for β-galactosidase-positive virus by the FDG assay (Fig. 4b). All of the transfections produced recombinant virus but with a substantial variation in efficiency depending on the treatment of the plasmid. Cutting the pAdlox plasmid at both ITRs (*Sfi*I) or at both ITRs plus cutting off the right ITR (*Sfi*I plus *Apa*I) produced equally high yields of recombinant virus. Cutting the plasmid with *Sca*I such that the ITRs remained buried in plasmid sequences reduced the yield to 17% of *Sfi*I-cut plasmid, and circular plasmid produced the least amount of recombinant virus, at about 4% of the rate of plasmid cleaved at the ITRs.

Next we used transfection to introduce the shuttle plasmid and compared infection with transfection to introduce Ψ5 DNA. We transfected cells with the shuttle vector and infected them with Ψ5 virus at various times relative to the infection, and we cotransfected one sample as before (see Materials and Methods for details). We used the virus produced from each sample to infect CRE8 cells and performed a restriction analysis on the packaged DNA to monitor the ratios of the donor and recombinant viruses (Fig. 5). Cotransfection of Ψ5 and pAdlox β-gal produced a virtually donor-free stock of recom-

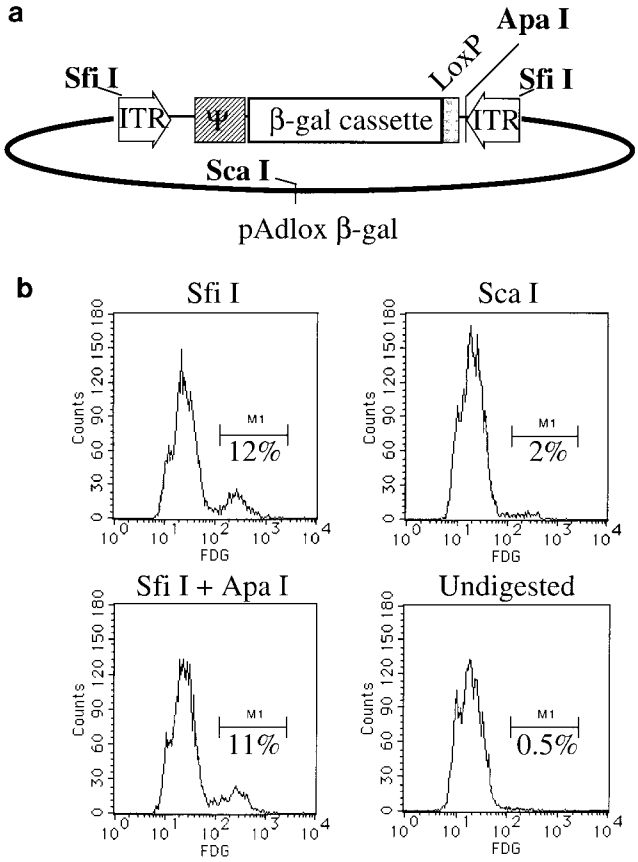

FIG. 4. (a) The pAdlox β-gal plasmid and the restriction enzyme cleavage sites used to assess the effect of shuttle vector topology on recombination. Cleavage with *Sfi*I excises a fragment capable of replicating in adenovirus-infected cells. Cleavage with *Apa*I in addition to *Sfi*I cuts off the right origin of replication, and cleavage with *Sca*I linearizes the plasmid but leaves the origins attached to bacterial DNA. (b) FDG analysis of recombination between Ψ5 and pAdlox cleaved in various ways. For quantitation, only the live single cells were used. The signal under the M1 bar is from the β-galactosidase-expressing cells.

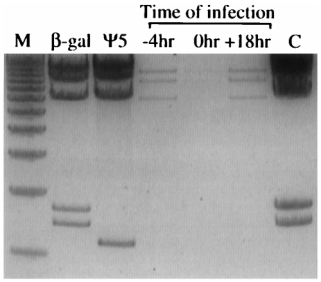

FIG. 5. Comparison of infection versus transfection as a means to introduce Ψ5 DNA for recombination. The lanes marked β-gal and Ψ5 are digests of Adβ-gal and Ψ5 DNAs to show the positions of the distinctive fragments. Lanes marked −4hr, 0hr, and 18hr are digests of viral DNA from infection of Ψ5 virus at various times relative to transfection of pAdlox β-gal; the sample marked C resulted from cotransfection of Ψ5 and pAdlox β-gal DNAs (see Materials and Methods for details). Packaged DNA from infection into CRE8 cells was digested with *Bsa*BI in all cases. The lane marked M is the 1-kb ladder DNA.

binant virus in 10 days. In contrast, infection of the donor virus combined with transfection of the shuttle plasmid produced less recombinant virus and a significant amount of donor contamination in a similar time frame.

In the foregoing experiment, there was very little donor virus DNA in cotransfected samples after the second passage through CRE8 cells. To determine more precisely how effectively the donor virus was removed during successive passages through CRE8 cells, we took the virus mixture from the cotransfection and passed it two more times through CRE8 cells, making lysates at each passage. Lysates which were stained with X-Gal and neutral red to distinguish the recombinant virus plaques from Ψ5 or mutant viruses were then diluted and infected into 293 cells for plaque analyses (Table 2). The initial transfection (passage 1) contained 30% Ψ5 virus. One passage through CRE8 cells reduced the Ψ5 virus to 3%, in agreement with the restriction pattern seen in Fig. 5. A further passage through CRE8 cells reduced the concentration of donor virus to 0.2%.

TABLE 2. Reduction of Ψ5 donor virus by passage through CRE8 cells

Passage no.	No. of blue plaques	No. of clear plaques	% Donor
P1	2×10^7	6×10^6	30
P2	1.5×10^8	5×10^6	3
P3	1×10^8	2×10^5	0.2

Gutless virus construction. Since the selection against Ψ5 functions in *cis*, it should be a good helper virus. Therefore, we decided to use Ψ5 to complement growth of an adenovirus vector which was missing all viral genes, a so-called gutless virus. Once again we used Ψ5 virus, CRE8 cells, and a shuttle plasmid in an interacting fashion (Fig. 6). The shuttle plasmid carrying the gutless virus genome, pAβ, was based on pCMV Ad (see Materials and Methods). It contained two ITRs with a packaging sequence in the left end, a β-galactosidase expression cassette as a marker, and a large piece of λ phage DNA as a nonfunctional substitute for the missing adenovirus DNA (Fig. 1b). In initial experiments, we determined the best scheme to turn pAβ plasmid into a virus by examining two sets of options, using either virus or viral DNA as a source of Ψ5 genomes and using either circular pAβ or pAβ cleaved at the ITRs as a source of the gutless genomes. The best combination was obtained by using cotransfection of the Ψ5 DNA with *Sfi*I-digested pAβ DNA, exactly the same conditions as we used for constructing E1-substituted viruses (data not shown).

Next, we tried to generate enough gutless virus for characterization. We cotransfected gutless plasmid with Ψ5 viral DNA into CRE8 cells, overlaid the cells with agar, and stained them for β-galactosidase activity at 7 days. About 90% of the approximately 50 plaques per dish turned blue, indicating the probable presence of the Aβ gutless virus. We amplified the viruses from these plaques and restriction mapped their DNAs. All of the viruses that we amplified appeared to be derivatives of Ψ5. A fragment that should have contained the left ITR, first *loxP* site, and packaging site of Ψ5 comigrated

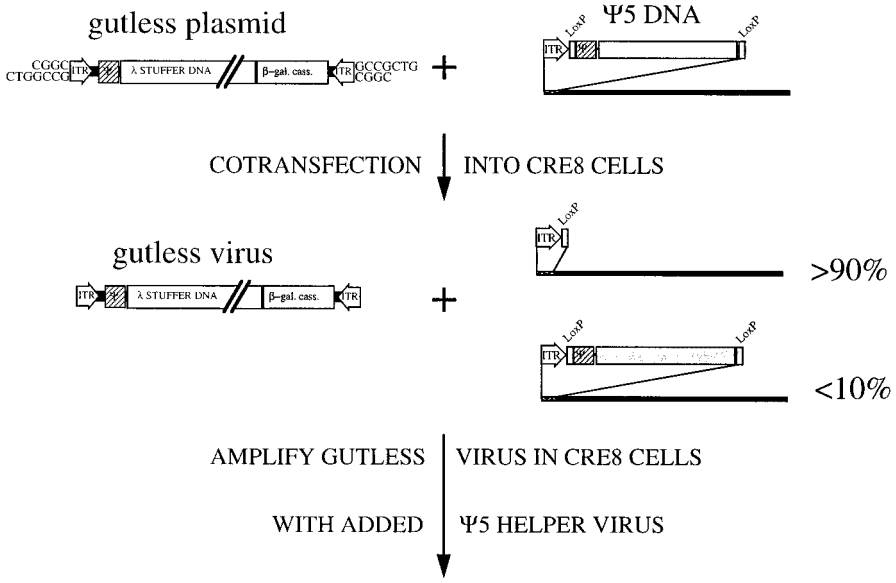

FIG. 6. Construction of a gutless adenovirus vector. In the first step, shuttle plasmid cleaved at the ITR is transfected into CRE8 cells with Ψ5 DNA. The proteins from Ψ5 convert the shuttle plasmid DNA to a molecule which is replicated by adenovirus DNA polymerase. The gutless virus is then encapsidated into adenovirus capsids. Several rounds of growth are necessary to amplify the gutless virus. At each round, more Ψ5 virus is added to ensure that all cells contain the helper virus.

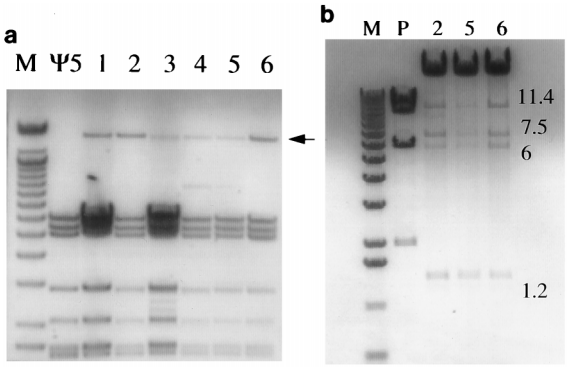

FIG. 7. (a) Restriction analysis of packaged gutless and Ψ5 DNA from infected CRE8 cells. The DNA was digested with *Bgl*II. There are no *Bgl*II sites in the loxAβ gutless virus. Lanes: M, 1-kb ladder plus 16.5- and 33.5-kb fragments; Ψ5, Ψ5 DNA; 1 to 6, isolates of loxAβ plus Ψ5. The arrow marks the position of loxAβ DNA. (b) Restriction analysis of loxAβ and Ψ5 DNAs with *Cla*I. Ψ5 DNA contains one site at base 1473. The predicted sizes of the loxAβ fragments are 0.5, 1.2, 6, 7.5, and 11.4 kb. Lanes: M, 1-kb ladder; P, ploxAβ cut with *Cla*I; 2, 5, and 6, isolates 2, 5, and 6 from panel a cut with *Cla*I. (Many of the bands from ploxAβ do not match their cognate bands in loxAβ, as the *Cla*I sites in the plasmid are methylated.)

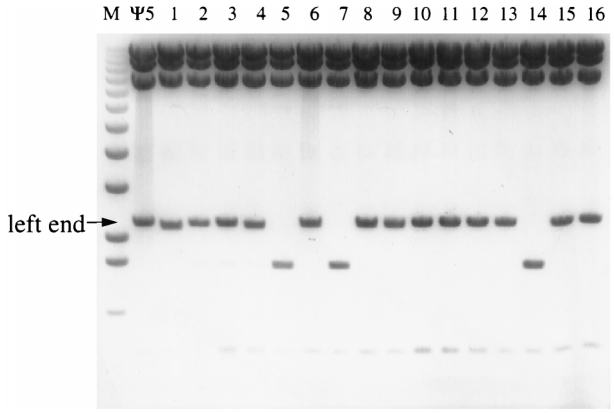

FIG. 8. Restriction analysis of Ψ5 mutants with *Bsa*BI. The position of the left-end fragment from Ψ5 is marked. The DNA was from 16 plaque isolates purified on 293 cells. Lane M, size markers.

instead with the equivalent fragment from pAβ, while all of the rest of the fragments were consistent with Ψ5 (data not shown). Thus, it appeared that pAβ and Ψ5 had recombined through homologous sequences somewhere between the two *loxP* sites in Ψ5. Since there are no *loxP* sites in pAβ, such a recombinant would have only one *loxP* site, giving the recombinant a strong advantage over Ψ5 for growth in CRE8 cells. To prevent this, we added a *loxP* site to pAβ between the left ITR and the packaging sequence, making ploxAβ (Fig. 1). Any recombinant would then be subjected to the same selection as Ψ5.

After cotransfecting *Sfi*I-digested ploxAβ DNA and Ψ5 viral DNA and staining for β-galactosidase activity as described above, we selected 12 blue plaques and amplified the loxAβ gutless virus on CRE8 cells by successive passage of the virus onto 10^4, 10^5, 10^6, and finally 10^7 cells. At each step, we added enough Ψ5 virus (multiplicity of infection of 5) to ensure that all of the cells were infected by the helper virus. Restriction analysis of packaged DNA from CRE8 cells infected with a portion of the virus from the 10^7 cell lysates showed that 10 of 12 contained an appreciable amount of loxAβ DNA (data for the first six are shown in Fig. 7a). The amount of loxAβ DNA varied from a few percent to 20% of the total amount of virus. Two of the isolates were passaged further by infection into CRE8 cells without supplemental Ψ5 virus. The amount of loxAβ DNA never increased above 20% of the amount of the helper virus. The yield of loxAβ virus was about 50 infectious particles per cell by the FDG assay.

To assess the integrity of the loxAβ virus, we analyzed fragments produced from a *Cla*I digestion of several of the isolates (Fig. 7b). *Cla*I digestion produced five major fragments from loxAβ, all of which migrated at their predicted sizes. We confirmed the loxAβ virus would transduce β-galactosidase activity into 293 and CV1 cells (data not shown).

Isolates 1 and 3 shown in Fig. 7a contained a large amount of helper virus DNA, indicating that Ψ5 had escaped selection. To determine the nature of the Ψ5 virus after the transfection and amplification process, we plaque purified viruses from these samples, taking eight plaques for each isolate, from which we prepared DNA. Restriction analyses of these DNAs demonstrate that many contain deletions while some might be intact (Fig. 8). Taking representative viruses, we amplified two segments containing the *loxP* sites by PCR and then sequenced the PCR products. The left *loxP* sites were intact. However, in all cases, the right *loxP* site was missing (data not shown).

To further analyze the stability of Ψ5 in CRE8 cells, we passaged Ψ5 virus through CRE8 cells. After eight passages, the virus began to grow well. Once again, we plaque purified isolates from the passaged virus and subjected the DNA to restriction analyses and sequencing. Of 10 viruses, 1 was still Ψ5 and the rest had deletions of one of the *loxP* sites (data not shown).

DISCUSSION

We have developed two methods for constructing recombinant adenoviruses by using Cre-*lox* recombination. Both methods rely on a *cis*-acting negative selection against an E1-deleted adenovirus that we call Ψ5 (Fig. 1a). The selection operates through a pair of directly repeated *loxP* sites flanking the packaging site. Cre recombinase catalyzes recombination between these *loxP* sites, excising the packaging site (Fig. 3). Such a deletion renders the affected viral chromosome unusable for packaging but leaves all other viral functions intact (15). These properties make Ψ5 an excellent helper virus for making recombinant adenoviruses.

Since high selection efficiency was critical for the success of our virus-building plans, we performed a detailed analysis of Ψ5 growth (Fig. 2a). When Ψ5 was grown in cells making Cre recombinase, over 90% of the molecules were recombined, and the yield of Ψ5 virus was about 10% of the yield of the unselected Ψ5 virus. The circular recombination product was present at a lower concentration than the deleted viral chromosome. The fact that the excised fragment was visible at all indicates that recombination was active during DNA replication. The relatively small quantity of the excised fragment may suggest that substantial recombination occurred before DNA replication. Replication of Ψ5 DNA was normal under selective conditions. These results clearly show that *lox* sites, Cre recombinase, and recombination do not impair adenovirus replication, in agreement with previous findings (1). Further, the negative selection was apparently by a mechanism of preferential packaging resulting from the deletion of the packaging site.

We confirmed that the selection efficiency was sufficient to remove Ψ5 from a mixture of two E1-substituted viruses (Fig.

2b and Table 2). Having two viruses in the same cell raises questions about the genetic stability of the selection, since adenoviruses recombine at a high rate. Recombination between Ψ5 and a viral vector without flanking *loxP* sites could produce a version of Ψ5 which would escape selection. Despite this, the negative selection was able to rapidly remove Ψ5 from a mixture containing another E1-substituted adenovirus.

Apart from selection efficiency, the yield of recombinant vectors is controlled by the conversion rate of transfected DNA into replicating adenovirus DNA. Two sets of findings bear on this process. First, from highest to lowest, the transfection efficiencies for various adenovirus DNAs are the following: viral DNA with terminal protein attached, protease-treated viral DNA, plasmid DNA cleaved at the ends of the ITRs, and circular DNAs or DNAs cleaved distal to the ITRs (5, 14, 21, 26). Second, a plasmid-derived DNA bounded by ITRs will replicate to 10,000 copies per cell if cotransfected with viral DNA but will replicate poorly if the viral DNA is introduced by infection (14). Thus, the yield of viral vectors was highest when we transfected shuttle plasmids that were cleaved next to the ITRs (Fig. 4b and Table 1). Similarly, the yield was higher with protease-treated viral DNA than with pBHG10 plasmid as the donor DNA (Table 1). Finally, cotransfection of shuttle plasmid with Ψ5 DNA proved more efficient than transfection of shuttle DNA and infection with Ψ5 virus at producing E1-substituted or gutless viruses (Fig. 5 and data not shown).

The final factor affecting the yield of E1-substituted viruses was the topology of the shuttle plasmid. We found that shuttle vector linearized at a site distal to the adenovirus sequences produced more recombinants than circular vector (Fig. 4b; compare uncut to *Sca*I cut). One explanation of these data is that the Cre-*lox* recombination reaction should be sensitive to the topology of the shuttle vector. The products of recombination between linear shuttle vector and Ψ5 DNA are two molecules, each with one *loxP* site. The product of recombination between a circular shuttle vector and Ψ5 DNA is a single molecule with two *loxP* sites. Chromosomes with two *loxP* sites are unstable, as they can rapidly recombine back to the two starting molecules by intramolecular recombination. Recombinants with single *loxP* sites are more stable, since their reversion occurs by much slower intermolecular recombination.

Applying these findings, we devised a plan to create E1-substituted viruses with high efficiency (Fig. 3). Ψ5 serves as a donor virus contributing the viral backbone to the new virus. A shuttle plasmid (pAdlox) cleaved at the ITRs provides the recombinant gene(s) and a packaging site without flanking *loxP* sites. CRE8 cells supply Cre recombinase, which both recombines the shuttle and donor vectors and eliminates the Ψ5 virus. As the new viruses produced by this arrangement have only one *loxP* site, they can rapidly overgrow Ψ5 (Table 2).

There are now several improved methods for making E1-substituted recombinant adenoviruses. Two such methods are driven by negative selection schemes. One method also uses a selection in *cis*, based on a packaging site deletion (3). The second uses a selection in *trans* against herpes virus thymidine kinase to remove the donor virus (17). Both of these methods use homologous recombination to construct the recombinant viruses. Cre-*lox*-mediated recombination has also been used to construct herpesviruses in an in vitro recombination scheme based on negative selection in *trans* (9). Fundamentally different approaches using reconstruction of the entire virus by homologous recombination either as yeast artificial chromosomes, as plasmids in bacteria, or as a virus in 293 cells have also been used to create recombinant adenoviruses (5, 18, 21).

The chief advantages of using the technique described here is that it is simple and highly efficient (Table 1). The major problem in using viral DNA as a source of donor DNA is having to purify the recombinants away from the donor virus. In this method, the purification was accomplished during amplification of the recombinant virus by a tight negative selection (Fig. 2b and Table 2). A secondary consideration in the choice of DNA for recombination is that plasmid DNA is much easier to prepare than viral DNA. While viral DNA is certainly more difficult to purify, it is also more stable, as the viral genome is under continuous selection during growth. In contrast, the viral sequences in plasmids are unselected. Finally, Cre-*lox* recombination enables the use of a small shuttle vector which is extremely easy to manipulate.

In our next method, we created an adenovirus vector which was missing all viral genes, or a gutless virus. The overall process was quite similar to one that we used for making E1-substituted viruses except that Ψ5 served as a helper virus (Fig. 6). Because this method relies on a genetic selection to remove the helper virus, the whole packaging capacity of adenovirus can be used for recombinant DNA. Additionally, the biological separation can easily be adapted for large-scale production of virus for use in gene therapy. In contrast, two recent reports have detailed methods for producing gutless viruses based on CsCl density gradient separations to remove the helper virus, a process which is difficult and expensive to scale up (7, 19).

Fundamentally, there should be two factors which limit the purity of gutless virus produced with a *cis*-selected helper virus. First is the stability and efficiency of selection against the helper virus. Second is the ability of the gutless virus to be replicated and packaged by the helper virus. From our data, it appears that neither the helper nor the gutless virus performed optimally.

Homologous recombination appeared to be one source of instability. While recombination between Ψ5 and a second virus was not a problem with E1-substituted viruses, such a recombinant did take over in the gutless virus case. We nullified the effects of recombination by placing a *loxP* site in the gutless virus before the packaging site, making ploxAβ (Fig. 1). Any recombination in the packaging sequence between the two viruses now regenerates the initial molecules.

A second process that allowed Ψ5 to escape selection was mutation of the *loxP* sites. We found that Ψ5 virus mutated by deleting one of the two *loxP* sites. Since the Cre-lox-based selection need be applied only in the final growth of the gutless virus, this instability is not a problem. The simple solution is to perform initial growth of a gutless vector in 293 cells, amplifying the gutless virus by using a positive selection based on a marker gene in the gutless virus.

Low yield of the gutless virus adds to any problem with the stability of the helper virus. This may explain why the stability of Ψ5 was not a problem in creating E1-substituted viruses but was a problem for making gutless viruses. The E1-substituted viruses have everything needed for viral growth in 293 cells. Even with Ψ5, the yield of gutless virus from each cell was still substantially less than that of a wild-type virus. Our gutless virus has the minimal sequences needed for replication and packaging of an adenovirus DNA, similar to the plasmids used in two other studies (7, 19). In one of these studies, a dystrophin-bearing gutless virus similar in size to our virus also produced little virus per cell (19). The simplest interpretation of these data is that both gutless viruses are deficient in some function. The deficit could be in any function requiring a *cis*-active component, such as replication, packaging, removal of the viral core proteins, directing the viral DNA to a proper location, stabilizing the DNA in some fashion, or a structural role of DNA in viral particles.

A likely source of problems is in DNA packaging. There could easily be auxiliary sequences that increase packaging efficiency. Alternatively, since the lower limit for packaging has yet to be defined, it is possible that there is a strong effect of length on adenovirus packaging. This notion is especially credible. Both our loxAβ virus and the dystrophin gutless virus are significantly smaller than normal, 75% or less of the size of the wild type. While these viruses were stable, a much smaller virus carrying only a *lacZ* cassette was extremely unstable, adding length by multimerizing or recombining with the helper (7). Thus, low yields and evidence of pressure to increase chromosomal length could indicate a strong preference for packaging full-length DNA. If this theory is correct, the low yield that we obtained could be easily corrected by inserting more stuffer DNA to increase the size of the gutless virus to 36 kb.

In summary, Cre-*lox* recombination is a powerful tool for genetic manipulation of adenoviruses. We present one application for constructing new E1-substituted adenoviruses which is an efficient alternative to the existing overlap recombination techniques. By this method, the viruses are almost pure without resorting to plaque purification. Thus, a working stock of virus can be produced for initial experiments in 10 days. In a second application, we demonstrate that a negatively selected helper virus can be used to support the growth of an adenovirus deleted of all viral coding sequences. This system, with some changes, should produce highly enriched gutless virus preparations in very large quantities for gene therapy.

ACKNOWLEDGMENTS

We thank Olivier Danos, Thomas Shenk, Richard Mulligan, Craig Jordan, Sean Sullivan, and John Forsayeth for critical reading of the manuscript or helpful discussions.

REFERENCES

1. **Anton, M., and F. L. Graham.** 1995. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. J. Virol. **69:**4600–4606.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, New York, N.Y.
3. **Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham.** 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. USA **91:**8802–8806.
4. **Bett, A. J., L. Prevec, and F. L. Graham.** 1993. Packaging capacity and stability of human adenovirus type 5 vector. J. Virol. **67:**5911–5921.
5. **Chartier, C., E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, and M. Mehtali.** 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J. Virol. **70:**4805–4810.
6. **Chinnadurai, G., S. Chinnadurai, and J. Brusca.** 1979. Physical mapping of a large-plaque mutation of adenovirus type 2. J. Virol. **32:**623–628.
7. **Fisher, K. J., H. Choi, J. Burda, S. Chen, and J. M. Wilson.** 1996. Recombinant adenoviruses deleted of all viral genes for gene therapy of cystic fibrosis. Virology **217:**11–22.
8. **Dai, Y., E. M. Schwarz, D. Gu, W. Zhang, N. Sarvetnick, and I. M. Verma.** 1995. Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. Proc. Natl. Acad. Sci. USA **92:**1401–1405.
9. **Gage, P. J., B. Sauer, M. Levine, and J. C. Glorioso.** 1992. A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. J. Virol. **66:**5509–5515.
10. **Grable, M., and P. Hearing.** 1990. Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. J. Virol. **64:**2047–2056.
11. **Grable, M., and P. Hearing.** 1992. *cis* and *trans* requirements for the selective packaging of adenovirus type 5 DNA. J. Virol. **66:**723–731.
12. **Graham, F. L., J. Smiley, W. C. Russel, and R. Narin.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
13a. **Hardy, S. F., M. Lewendowski, G. Martin, and Z. W. Hall.** Unpublished data.
14. **Hay, R. T., N. D. Stow, and I. M. McDougall.** 1984. Replication of adenovirus mini-chromosomes. J. Mol. Biol. **175:**493–510.
15. **Hearing, P., R. J. Samulski, W. L. Wishart, and T. Shenk.** 1987. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. J. Virol. **61:**2555–2558.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
17. **Imler, J. L., C. Chartier, A. Dieterle, D. Dreyer, M. Mehtali, and A. Pavirani.** 1995. An efficient procedure to select and recover recombinant adenovirus vectors. Gene Ther. **2:**263–268.
18. **Ketner, G., F. Spencer, S. Tugendriech, C. Connelly, and P. Hieter.** 1994. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. Proc. Natl. Acad. Sci. USA **91:**6186–6190.
19. **Kochanek, S., P. R. Clemens, K. Mitani, H. H. Chen, S. Chan, and C. T. Caskey.** 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galctosidase. Proc. Natl. Acad. Sci. USA **93:**5731–5736.
20. **McGrory, W. J., D. S. Basutista, and F. L. Graham.** 1988. Short Communications: a simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. Virology **163:**614–617.
21. **Miyake, S., M. Makimura, Y. Kanegae, S. Harada, Y. Sato, K. Takamori, C. Tokuda, and I. Saito.** 1996. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing full-length virus genome. Proc. Natl. Acad. Sci. USA **93:**1320–1324.
22. **Nolan, G. P., S. Fiering, J.-F. Nicolas, and L. A. Herzenberg.** 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc. Natl. Acad. Sci. USA **85:**2603–2607.
23. **Sauer, B.** 1987. Functional expression of the *cre-lox* site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2087–2096.
24. **Shenk, T.** 1996. Adenoviridae: the viruses and their replication, p. 2111–2148. *In* B. N. Fields et al. (ed.), Fields virology. Raven Press, New York, N.Y.
25. **Stow, N. D.** 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. J. Virol. **37:**171–180.
26. **Tamanoi, F., and B. W. Stillman.** 1982. Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. USA **79:**2221–2225.
27. **Thimmappaya, B., C. Weinberger, R. J. Schnider, and T. Shenk.** 1982. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell **31:**543–551.
28. **Yang, Y., Q. Li, H. C. J. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.
29. **Young, C. S. H., T. Shenk, and H. S. Ginsberg.** 1984. The genetic system, p. 125–172. *In* H. S. Ginsberg (ed.). The adenoviruses. Plenum Press, New York, N.Y.