

Efficient Generation of Recombinant Adenovirus Vectors by Homologous Recombination in *Escherichia coli*

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Received 18 January 1996/Accepted 12 April 1996

Despite recent technical improvements, the construction of recombinant adenovirus vectors remains a time-consuming procedure which requires extensive manipulations of the viral genome in both *Escherichia coli* and eukaryotic cells. This report describes a novel system based on the cloning and manipulation of the full-length adenovirus genome as a stable plasmid in *E. coli*, by using the bacterial homologous recombination machinery. The efficiency and flexibility of the method are illustrated by the cloning of the wild-type adenovirus type 5 genome, the insertion of a constitutive promoter upstream from the E3 region, the replacement of the E1 region by an exogenous expression cassette, and the deletion of the E1 region. All recombinant viral DNAs were shown to be fully infectious in permissive cells, and the modified E3 region or the inserted foreign gene was correctly expressed in the infected cells.

Adenoviruses are generally associated with benign pathologies in humans and are characterized by a number of features which make them particularly attractive as gene transfer vectors for gene therapy or immunization purposes (7, 15, 21, 35). Most vectors are based on the adenovirus type 5 (Ad5) backbone in which an expression cassette containing the foreign gene has been introduced in place of the early region 1 (E1) or early region 3 (E3). Viruses in which E1 has been deleted are defective for replication and are propagated in the human 293 complementation cells, providing the E1 products in *trans* (16). Methods to construct such recombinant adenoviruses are well documented (3, 14). They usually consist of a first step of subcloning of the exogenous expression cassette into a segment of the viral genome. The recombinant vector is then produced by the reconstitution of a complete viral DNA molecule through ligation *in vitro* between the segment and the viral genome, followed by transfection into permissive cells. Alternatively, cotransfection into the complementation cells of the viral genome and plasmid DNA molecules can generate the recombinant viruses by homologous recombination *in vivo*. These methods frequently generate a background of nonrecombinant viruses, and despite recent improvements (23), repeated screening of many plaques is sometimes required in order to isolate pure recombinant vectors.

Two alternative procedures in which no parental infectious viral genome is used have been described (5, 24, 29). One method is based on the use of overlapping Ad5 DNA sequences cloned in two bacterial plasmids (5). The first plasmid carries the total Ad5 genome with a deletion of the DNA packaging signal and part of the E1 region, with the left and right inverted terminal repeat (ITR) sequences directly covalently joined. The second plasmid contains the left-end viral sequences, including the packaging signal and a passenger gene in place of the E1 region. Cotransfection of these plasmids in 293 cells results in the production of infectious recombinant vectors by *in vivo* homologous recombination. The configuration of the first plasmid is however known to be unstable in *Escherichia coli* (13, 28, 31). Moreover, the introduction of

specific mutations or deletions in regions other than E1 requires tedious preliminary cloning steps in *E. coli*. The second alternative method is based on the manipulation of the full-length Ad genome as an infectious yeast artificial chromosome (YAC) (24). Targeted modifications of the viral genome are introduced by homologous recombination in yeast cells, and infectious virions are generated after transfection of the adenovirus genome, excised from the YAC vector, into appropriate cells. Although powerful, this method requires the use of an additional host (yeast) in which DNA yields are relatively low.

In the present study, we describe a novel procedure for the generation of recombinant adenovirus vectors that takes advantage of the highly efficient homologous recombination machinery of *E. coli* (6, 10, 11, 25, 30). We applied this method to clone the full-length Ad5 genome in one unique and stable infectious bacterial plasmid and to modify specifically several viral genetic regions. The production of vectors constitutively expressing the E3 region and/or bearing a human coagulation factor IX (hFIX) expression cassette in place of E1 is described in this paper as an example of the flexibility of this method.

The full-length 36-kb Ad5 genome was first cloned in a 2-kb plasmid by homologous recombination in *E. coli* between Ad5 virion DNA and a linear form of the pTG3601 plasmid (Fig. 1A; Table 1). pTG3601 was derived from the ppolyII plasmid (27) by insertion of 935- and 853-bp Ad5 DNA fragments amplified by PCR from the left and right ends of the Ad5 genome, respectively. Cotransformation of the *Bgl*III-linearized pTG3601 plasmid and Ad5 genomic DNA into *E. coli* BJ5183 *recBC sbcBC* (18) regenerated a stable circular pTG3602 plasmid containing the total Ad5 genome and conferring ampicillin resistance to the bacterium (Fig. 1). The frequency of recombinants was high, as estimated by the ratio between the number of ampicillin-resistant colonies obtained after cotransformation of *Bgl*III-digested pTG3601 and Ad5 DNA and the number of colonies obtained after transformation of the linearized pTG3601 plasmid alone. Depending on the experimental conditions, this ratio was between 5 and 61 (Table 1). This ratio indicates that the linearization of the plasmid prevents the growth of background colonies containing the parental vector. The efficiency of recombination could, however, not be significantly improved by altering the insert (I):vector (V) ratio (Table 1). One explanation might be that the size of the Ad5

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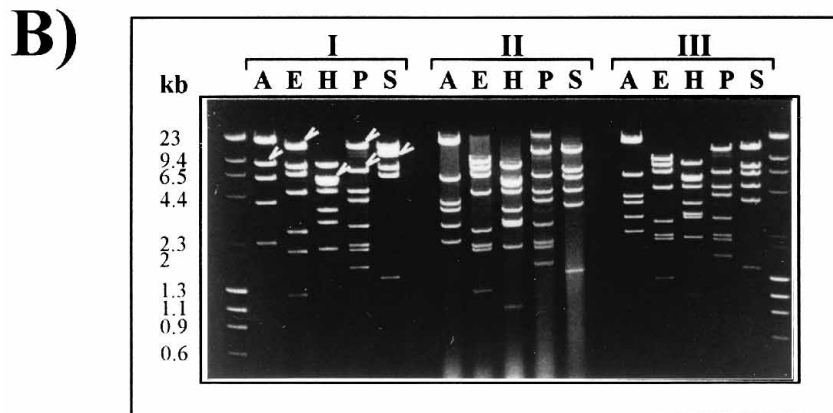
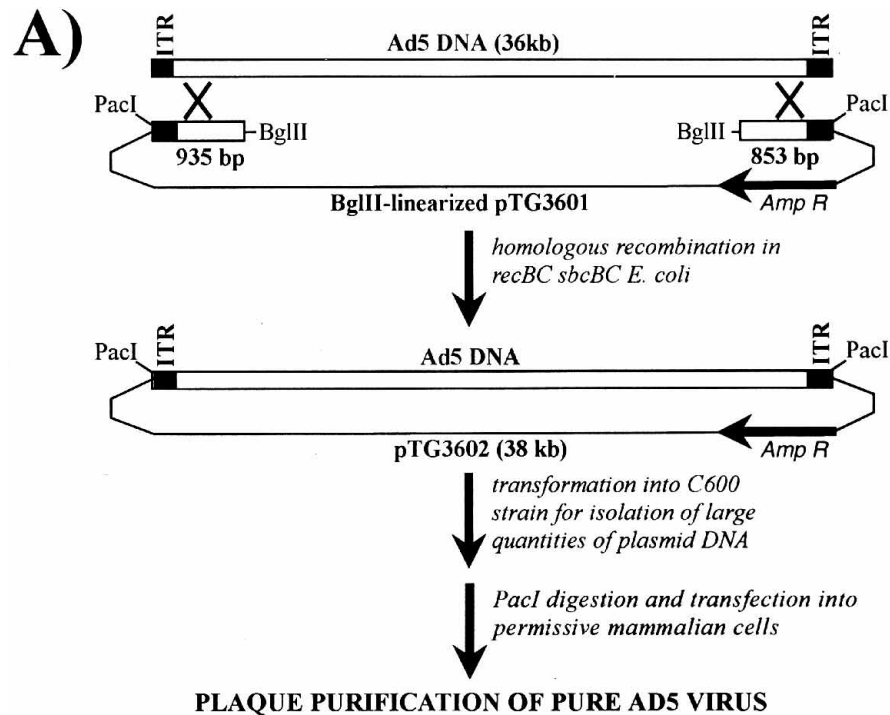


FIG. 1. Recombinational cloning of infectious full-length Ad5 genome in *E. coli*. (A) *E. coli* BJ5183 was cotransformed with Ad5 genomic DNA and a BglIII-linearized pTG3601 plasmid containing 935 and 853 bp from the left and right ends of Ad5, respectively. In vivo homologous recombination generates ampicillin-resistant colonies containing the 38-kb pTG3602 plasmid carrying the full-length Ad5 genome. Large-scale preparation of pTG3602 DNA was carried out in the C600 *E. coli* strain. Cloned Ad5 DNA is still fully infectious as shown by the development of virus plaques after transfection of permissive human cells with PacI-restricted pTG3602 DNA. (B) Restriction endonuclease analysis of pTG3602 plasmid DNA (lanes I), DNA purified from AdTG3602 virus produced by transfection of PacI-restricted pTG3602 DNA (lanes II), and wild-type Ad5 DNA (lanes III). Plasmid DNA (1 μ g), 10 μ l of viral DNA prepared according to the method of Hirt (20), or 1 μ g of purified Ad5 DNA was digested with *Afl*III (A), *Eco*RV (E), *Hind*III (H), *Pvu*I (P), and *Sph*I (S). Carets mark the positions of bands corresponding to the plasmid-Ad5 junctions. Size markers are indicated on the left.

genome (36 kb) constitutes a limiting factor for the efficiency of transformation. This possibility is supported by the observation that *E. coli* transformation is inhibited when increased amounts of Ad5 DNA are used (Table 1).

Eight bacterial colonies randomly isolated for further characterization were all shown by DNA restriction analysis to contain the expected plasmid (Table 1), suggesting that each recombination event occurred through homologous pairing of free viral DNA ends. However, plasmid DNA yields were low in BJ5183 cells, probably because of the formation of plasmid multimers (26). pTG3602 DNA was therefore retransformed

into the C600 bacterial strain (22) to isolate larger quantities of plasmid DNA. Extensive characterization of pTG3602 DNA by restriction endonuclease analysis failed to detect any visible DNA rearrangement (Fig. 1B). The stability of the 38-kb plasmid is presumably due to the insertion of the 2-kb ppolYII sequence between the two viral ITRs, avoiding the constitution of palindromic sequences (28, 31).

Two unique *Pac*I sites were introduced by PCR immediately upstream of the left ITR and downstream of the right ITR in the initial pTG3601 plasmid (Fig. 1A). Since *Pac*I is absent in Ad5 genomic DNA, *Pac*I digestion allows the precise excision

TABLE 1. Efficiency of recombinational cloning of full-length Ad5 genome

Vector pTG3601- <i>Bgl</i> II (ng)	Insert Ad5 DNA (ng)	I:V molar ratio ^a	No. of Amp ^r colonies	HR/V ^b
1	0		4	
	9.4	1	245	61
10	94	10	136	34
	0		64	
	94	1	627	10
	940	10	321	5

^a I, insert; V, vector.

^b The efficiency of homologous recombination (HR) is indicated by the HR/V ratio, calculated as the ratio of the number of colonies generated by the cotransfected vector and Ad5 insert DNA and the number of colonies generated by the vector alone.

of the full-length Ad5 genome from the pTG3602 plasmid. The infectivity of this pTG3602-derived Ad5 genome was demonstrated by calcium phosphate transfection (17) of 293 and A549 cells (34): large numbers of plaques were observed in cells transfected with the *Pac*I-restricted pTG3602 DNA, while the closed circular plasmid was unable to generate any viral plaques, confirming that at least one ITR extremity has to be in a free configuration to allow efficient adenovirus DNA replication (Table 2) (4, 19). These results show that the plasmid-derived Ad5 genome is fully infectious, with a specific infectivity of about 1/20 to 1/40 of that of Ad5 genomic DNA purified from wild-type virus particles (Table 2). Progeny virus recovered from independent pTG3602 plaques was amplified on 293 cells and further analyzed for growth characteristics, virus production yields, and DNA restriction patterns. In all

TABLE 2. Specific infectivity of cloned wild-type and recombinant Ad5 genomes^a

DNA	DNA concn	No. of plaques/6-cm dish	
		293 cells	A549 cells
Ad5	0.1	>50, >50	4, 5
	1	Lysed, lysed	23, 34
pTG3602	1	0, 0	0, 0
	5	0, 0	0, 0
pTG3602- <i>Pac</i> I	1	23, 33	1, 0
	5	Lysed, lysed	6, 10
pTG3604- <i>Pac</i> I	1	53	1
	5	Lysed	13
pTG3614- <i>Pac</i> I	1	20, 41	ND
	5	Lysed, lysed	ND
pTG3606- <i>Pac</i> I	1	52, 27	ND
	5	Lysed, lysed	ND
pTG3622- <i>Pac</i> I	1	17, 9	ND
	5	63, 60	ND
pTG3623- <i>Pac</i> I	1	17, 17	ND
	5	34, 22	ND

^a A549 and/or 293 cells were transfected with 0.1 and 1 μ g of Ad5 DNA or nonrestricted or *Pac*I-restricted plasmid DNA. Cells were overlaid with agar 15 h later, and plaques were then counted 14 days posttransfection. ND, not done (these viruses have E1 deletions and do not grow on A549 cells). Results from two independent experiments are shown (except for those for pTG3604-*Pac*I).

cases, pTG3602-derived adenovirus (AdTG3602) was indistinguishable from wild-type Ad5 (Fig. 1B).

Since construction of recombinant adenovirus vectors is usually a time-consuming procedure, we then tested whether a single-step replacement strategy exploiting the *E. coli* homologous recombination machinery could be designed to modify selectively any particular genetic region of Ad5 (Fig. 2). The viral region to be modified is first subcloned into a bacterial plasmid and the desired deletions, insertions, or mutations are performed by conventional molecular biology techniques (32). The modified segment is then purified and cotransformed into BJ5183 together with the appropriately restricted plasmid containing the full-length Ad5 genome (Fig. 2). Homologous recombination events between the free ends of the modified replacement fragment and their homologous viral sequences in the linearized vector eventually generate plasmids containing Ad5 genomes with the required modifications.

As an example, we derived from pTG3602 a new plasmid (pTG3604) containing a full-length Ad5 genome in which expression of E3 (36) was relieved from the control by E1 proteins and made constitutive by the introduction of the Rous sarcoma virus 3' long terminal repeat promoter sequences (LTR_{RSV}). LTR_{RSV} was inserted in front of E3 at nucleotide (nt) 28249 (throughout the manuscript, nucleotide numbers refer to positions on the Ad5 genome, according to reference 8) in a transfer plasmid (pTG1696) bearing an Ad5 segment from nt 21562 to the right-end ITR. This insertion site was chosen in order to maintain the integrity of the L4 mRNA polyadenylation signal which overlaps the E3 mRNA transcription initiation site (33). A *Bgl*II^(nt 24862-35211) fragment encompassing the modified E3 region was then isolated from pTG1696 and recombined in *E. coli* with pTG3602 previously linearized at nt position 27082 by *Spe*I digestion (Fig. 2A). *Spe*I cuts the Ad5 genome upstream of the targeted E3 region and leaves three regions of DNA homology with the pTG1696 *Bgl*II fragment: (i) 2,220 bp between the *Bgl*II^(nt 24862) and *Spe*I^(nt 27082) sites, (ii) 1,167 bp between *Spe*I^(nt 27082) and the 5' boundary (nt 28249) of LTR_{RSV}, and (iii) 7,623 bp between the 3' boundary (nt 27588) of LTR_{RSV} and the *Bgl*II^(nt 35211) site. As a consequence, homologous recombination should generate two types of plasmids (Fig. 2A): (i) the parental pTG3602 plasmid reconstituted by recombination between sequences upstream from the *Spe*I site and sequences located between *Spe*I and the E3 region and (ii) the expected pTG3604 plasmid containing the E3-modified genome produced by recombination between sequences upstream from *Spe*I and downstream from the E3 region. The radioactive screening of the ampicillin-resistant colonies with an LTR_{RSV} oligonucleotide probe confirmed this hypothesis since 18.5% of the 270 colonies were found to contain the pTG3604 plasmid (Table 3). DNA analysis performed on six randomly selected candidates confirmed that the cloned viral genome was genetically stable and full-length and contained a modified E3 region. Moreover, homologous recombination in BJ5183 was highly efficient since 270 ampicillin-resistant colonies were obtained after cotransformation with the linearized pTG3602 plasmid and the pTG1696 fragment, while only 2 colonies appeared with the linearized pTG3602 vector alone (Table 3).

Similar to E3, the E1 region can also be efficiently modified by homologous recombination in *E. coli*. As an example (Fig. 2B and C), the E1 region of Ad5 was either deleted or replaced by an expression cassette encoding hFIX (2). Replacement of E1 by hFIX was done by cotransformation of *E. coli* BJ5183 with a pTG3602 or pTG3604 plasmid linearized in E1 by *Cl*AI^(nt 918) digestion and an *Msc*I DNA fragment bearing the hFIX cDNA under the control of the mouse phosphoglycerate kinase gene

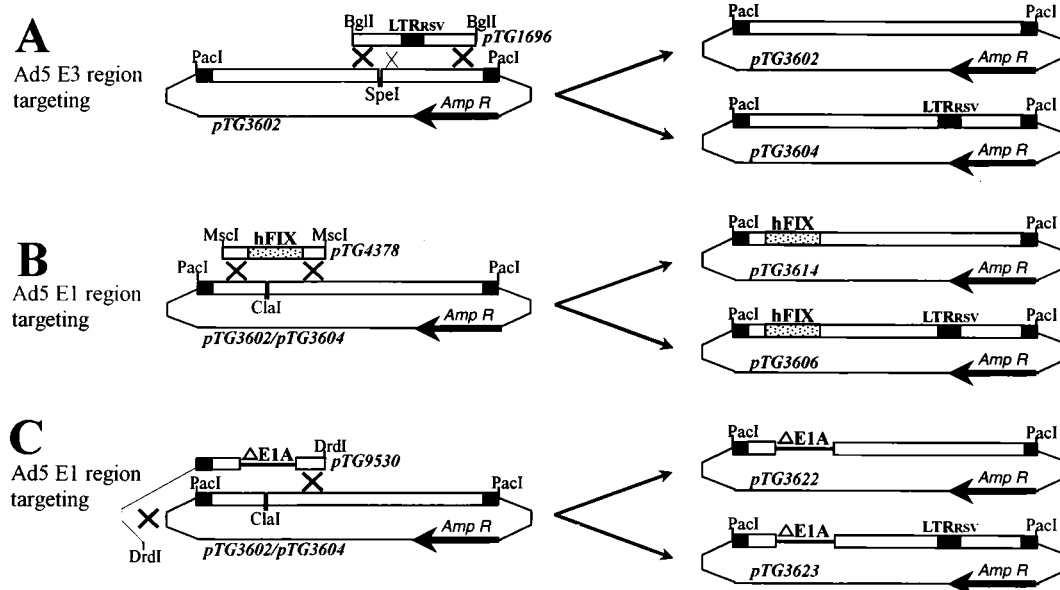


FIG. 2. Construction of recombinant adenovirus vectors by homologous recombination in *E. coli*. Several strategies of Ad5 genome manipulation are presented: insertion of a new regulatory sequence (LTR_{RSV} promoter) upstream of the Ad5 E3 region (A), replacement of the Ad5 E1 region by a foreign sequence (expression cassette for human coagulation factor IX) (B), and deletion of the Ad5 E1 region (C). ×, homologous recombination events required for the targeted modification of the viral DNA; ×, an homologous recombination event that reconstitutes the parental target vector (panel A). ITRs (closed boxes), Ad5 genomic DNA (open boxes), foreign sequences (stippled boxes), E1 deletion (thick line), plasmid sequences (thin lines), β-lactamase gene (arrows), and plasmid excision (labeled *PacI*) and linearization (labeled *Bgl*II, *Cla*I, and *Spe*I) restriction sites are also indicated.

promoter (1) and the simian virus 40 polyadenylation signal (Fig. 2B). The *MscI* insert was isolated from a transfer plasmid (pTG4378) containing the hFIX expression cassette in place of the E1 region (nt 459 to 3328), in an adenovirus DNA fragment corresponding to 17% of the left genomic sequence (nt 1 to 6241). Excision by *MscI* leaves upstream and downstream from the hFIX cassette 188 and 2,044 bp, respectively, of Ad5 sequences homologous to viral DNA in pTG3602 and pTG3604 (Fig. 2B). Cotransformation into BJ5183 generated only few ampicillin-resistant colonies (Table 3), probably as a consequence of the short length (188 bp) of sequence with DNA homology upstream of the *Cla*I site (see below). However, linearization of target vectors in the E1 region significantly increased the frequency of colonies containing plasmids

in which E1 was replaced by the hFIX expression cassette: 1 positive colony of 2 and 4 positive colonies of 17 identified for the pTG3602- and pTG3604-derived vectors, respectively (Table 3). The negative colonies contained the background pTG3602 and pTG3604 parental vectors, probably corresponding to residual uncut vectors.

We tested whether increasing the length of the region with DNA homology upstream of the E1-replacement cassette might improve the homologous recombination efficiency (30). We used a transfer plasmid (pTG9530) containing an Ad5 left-end region (nt 1 to 5788) deleted from part of the E1 sequences (nt 459 to 3328) in the same polyII plasmid backbone as pTG3602. A *DrdI* fragment isolated from pTG9530 was cointroduced into bacteria along with the *Cla*I-restricted pTG3602 or pTG3604 target vector (Fig. 2C). The sequence of DNA homology upstream of the *Cla*I site was 2,244 bp long (compared with 188 bp in the previous experiment), and this increase in length dramatically affected the HR/V ratio, which increased to 101/1 and 569/48 for the pTG3602- and pTG3604-derived vectors, respectively (Table 3). Analysis of six randomly picked colonies showed the presence of the expected pTG3622 (full-length Ad5 deleted from E1) and pTG3623 (full-length Ad5 deleted from E1 and with a constitutive E3 region) recombinant plasmids.

These experiments demonstrate that the replacement or the deletion of regulatory or structural viral genes can be easily achieved by homologous recombination in *E. coli*. The advantages of using *E. coli* instead of yeast cells (24) include higher transfection efficiency, higher growth rates, and higher plasmid yields. Moreover, the *E. coli* single-step replacement strategy is technically more straightforward than the two-steps replacement strategy classically used to target a segment of yeast DNA. We observed that the frequency of homologous recombination events and the efficiency of recovery of the expected recombinants could vary depending on the design of the DNA

TABLE 3. Efficiency of targeted modifications of the cloned Ad5 genome^a

Insert	Vector	HR/V ^b	No. of positive colonies/total no. of colonies (plasmid)
pTG1696- <i>Bgl</i> II	pTG3602- <i>Spe</i> I	270/2	50/270 (pTG3604)
pTG4378- <i>Msc</i> I	pTG3602- <i>Cla</i> I	2/0	1/2 (pTG3614)
	pTG3604- <i>Cla</i> I	17/19	4/17 (pTG3606)
pTG9530- <i>Drd</i> I	pTG3602- <i>Cla</i> I	101/1	6/6 (pTG3622)
	pTG3604- <i>Cla</i> I	569/48	6/6 (pTG3623)

^a Plasmids carrying wild-type (pTG3602) or E3-modified (AdTG3604) Ad5 genomes were digested by *Spe*I for E3 region targeting or by *Cla*I for E1 region targeting and transformed into *E. coli* BJ5183 either alone or together with a 10-fold molar excess of the purified replacement insert. Positive colonies containing plasmids with the modified full-length Ad5 genomes were identified by radioactive screening or with a plasmid miniprep.

^b Number of colonies generated by the cotransfected vector and insert per the number of colonies generated by the vector alone.

transfer strategy. In the described examples (Fig. 2B and C; Table 3), the frequency of homologous recombination could be enhanced 10- to 100-fold by increasing the length of the region with DNA homology. Moreover, the yield of positive recombinants was higher when the vector was linearized precisely in the targeted region, as in the E1 replacement experiments (Fig. 2B and C). Modification of the E3 region was done with a target vector linearized at a *SpeI* site located 1,167 bp upstream from E3. As a consequence, two types of plasmids were obtained: the parental pTG3602 vector and the E3-modified Ad5 genome (Fig. 2A). The same phenomenon was observed in deletion experiments targeting the fiber gene or the E4 region which is located 4.8 or 5.8 kb downstream of the *SpeI* site, respectively: the frequency of correct recombination events was found to be inversely proportional to the distance between the targeted region and the *SpeI* site (unpublished observations). The percentage of positive colonies was nevertheless always >1%, and the identification of these colonies by classical screening techniques was straightforward.

Transfection of *PacI*-restricted pTG3604, pTG3606, pTG3614, pTG3622, and pTG3623 plasmid DNA into 293 cells allowed the production of viral plaques with efficiencies similar to that obtained with pTG3602 (Table 2). In all cases, transfection of as little as 1 μ g of viral DNA was sufficient to generate enough viral plaques for virus propagation and purification; the specific infectivity was around 30 plaques per μ g of linearized adenovirus DNA in 293 cells (Table 2). As a comparison, adenovirus DNA excised from a YAC was shown to generate 2 to 10 plaques per μ g of total yeast DNA, corresponding to a calculated specific infectivity of 100 to 500 plaques per μ g of viral DNA (24). The method recently described by Bett et al. (5) and McGrory et al. (29) generates 4 to 8 plaques per μ g of transfected plasmid DNA. In the latter case, the transfer plasmid is cotransfected with a circular plasmid bearing the Ad5 genome, which is in a relatively unstable configuration and is less infectious than linear DNA (12, 13). The procedures described here and by Ketner et al. (24) are based on prior manipulation of the full-length viral genome contained in single molecules of stable YACs or plasmids. Transfection into permissive eukaryotic cells of Ad5 genome excised from YAC or plasmid sequences allows the systematic recovery of pure virus plaques. Identification of the positive recombinant viruses by plaque screening and isolation of pure virus stocks by repeated plaque purifications are therefore not required for these methods. As a consequence, the construction of recombinant vectors by the described procedure is usually faster than that by previous methods; as an example, production of pure plaques of a vector in which E1 is deleted with pTG3602 and the E1-transfer plasmid as starting materials requires at most 20 days compared with at least 6 weeks for methods in which similar starting materials (Ad5 DNA and E1-transfer plasmid) are cotransfected in 293 cells (3, 14).

The analysis of the phenotype of all plasmid-derived viruses confirmed that the selected modifications were correctly and stably introduced into the viral genomes. As an illustration, we have shown that expression of E3 in AdTG3606 is no longer controlled by E1 but is driven by the constitutive LTR_{RSV} promoter sequences inserted upstream from the E3 coding region (Fig. 3A). 293 and A549 cells were infected with wild-type Ad5 or E1-deleted AdTG3606 or AdTG3614 viruses at multiplicities of infection of 10 (data not shown) and 100 (Fig. 3A). Radioimmunoprecipitation of the cell extracts with monoclonal antibody Tw1.3 (9) directed against the E3-encoded gp19K protein showed an expression of the viral glycoprotein in both cell lines infected with either Ad5 or AdTG3606. In contrast, infection with a virus with an E1 de-

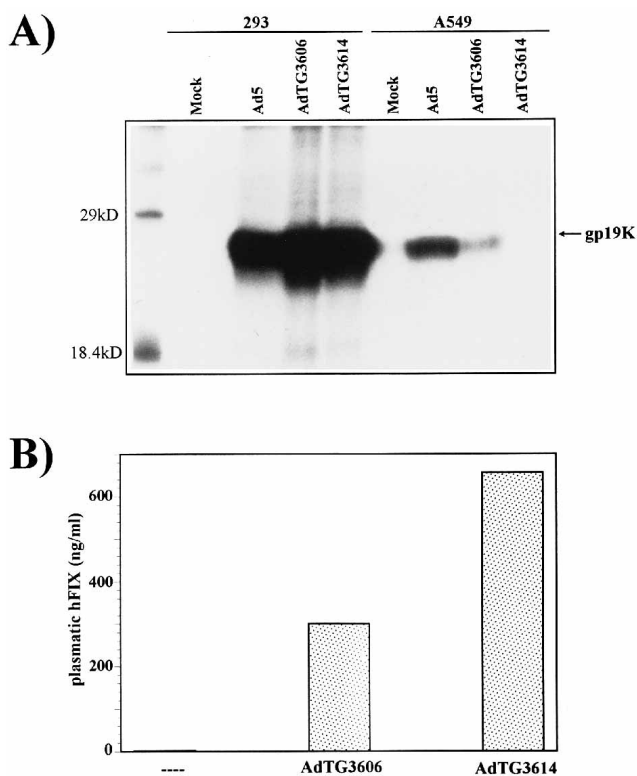


FIG. 3. Foreign sequences introduced into recombinant viruses by in vivo homologous recombination are functional. (A) Insertion of the LTR_{RSV} promoter sequences upstream of the Ad5 E3 region confers constitutive expression to gp19K. 293 and A549 cells were mock infected or infected for 24 h with AdTG3606, AdTG3614, or wild-type Ad5. Total proteins labeled with [³⁵S]methionine/cysteine were immunoprecipitated with MAb Tw1.3, an anti-gp19K monoclonal antibody, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (B) purified AdTG3606 or AdTG3614 recombinant vector in which the E1 region was replaced by an expression cassette for hFIX was injected into the tail vein of three C57BL/6 mice. Serum was recovered at 3 days postinjection and analyzed by enzyme-linked immunosorbent assay for the plasmatic concentration in human factor IX. The means of data for each set of animals is shown. This first lane (---) shows the results for a control, untreated mouse. In panel A, size markers (in kilodaltons [kD]) are indicated on the left.

letion and carrying wild-type E3 sequences (AdTG3614) led to gp19K expression only in the E1-expressing 293 cells (Fig. 3A). In addition, the intravenous administration of AdTG3606 or AdTG3614 containing an hFIX expression cassette in place of E1 led to high concentrations of hFIX in C57BL/6 mice: 3 days after virus injection, mean levels of 300 and 650 ng/ml were detected in the sera of the animals treated with AdTG3606 and AdTG3614, respectively (Fig. 3B).

The procedure described in this report allows the rapid and efficient cloning and manipulation of full-length infectious adenovirus genomes in bacterial plasmids. This method combines the powerful genetic engineering techniques that are available in *E. coli* and the ability of this microorganism to recombine homologous sequences at a high frequency (6, 10, 11, 25, 30). The advantages of this technology are multiple: (i) all cloning and, more importantly, recombination steps are carried out in *E. coli*, (ii) the frequency of bacterial colonies containing the plasmid with the modified adenovirus genome is very high, (iii) any genetic region of the viral genome can be specifically modified or deleted if appropriate restriction sites are available, (iv) plasmids containing the full-length adenovirus genomes can be introduced into appropriate bacterial strains for production of large amounts of viral DNA, and (v) transfection

of excised recombinant adenovirus DNA into permissive human cells generates plaques containing only pure virus particles. We demonstrate that full-length recombinant adenovirus genomes modified in both E1 and E3 regions can be efficiently generated and that their rescue as pure viral particles was guaranteed in transfected 293 cells. We similarly produced adenoviruses with either deletions of or modifications in the fiber gene, the E2A gene, or the E4 region (unpublished data). The production of even-further-crippled viral vectors is theoretically possible with the same technology, provided that adequate restriction sites are available for Ad5 DNA linearization. The presence of the *Clal*, *BamHI*, and *SpeI* sites located at positions 918, 21562, and 27082, respectively, already allows efficient modifications of most of the viral genes. However, improvement of this approach is possible by the introduction of new unique sites at other locations. Investigation of these sites is currently in progress.

We thank J. W. Yewdell for kindly providing the MAb Tw1.3 anti-gp19K monoclonal antibody. We also thank H. Schultz, D. Ali-Hadji, P. Kopp, and I. Mauclair for excellent technical assistance and M. Courtney and M. Lusky for critical reading of the manuscript.

This work was supported in part by the Association Française contre la Mucoviscidose and the Association Française contre les Myopathies.

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