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Efficient Selection of Recombinant Adenoviruses by Vectors That Express β-Galactosidase

JEROME SCHAACK,* STEPHEN LANGER, AND XIAOLING GUO

Department of Microbiology, Program in Molecular Biology, and University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Adenovirus serotype 5 vectors which contain the *Escherichia coli* β-galactosidase gene driven by the cytomegalovirus immediate-early promoter as a screenable marker have been made and successfully used in the construction of recombinant adenoviruses. The β-galactosidase gene has been introduced into viruses in which the E3 region is maintained or deleted and in which the *cis*-acting packaging sequence has been reiterated at the right end of the chromosome. A unique *Bst*BI site has been introduced 3′ of the β-galactosidase gene. Cotransfection of *Bst*BI-digested vector DNA and a plasmid containing the left end of the viral chromosome followed by staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) results in clear plaques when overlap recombination has occurred and blue plaques when ligation of the viral arms has occurred within the host cell. The β-galactosidase-expressing viruses grow to lower titers than do the parental viruses, leading to a relative growth advantage for viruses resulting from overlap recombination. Combined with color selection based on the β-galactosidase gene, this system permits efficient production and selection of recombinant viruses after cotransfection of *Bst*BI-digested viral DNA with a plasmid including left-end viral sequences and the gene of interest. The β-galactosidase-expressing viral DNAs were used to construct viruses containing *Bst*BI sites on either side of the *cis*-acting packaging element as a means of testing their utility.

Adenovirus vectors have become important tools for transduction experiments as well as for gene therapy. The size of the adenovirus genome makes introduction of foreign genes in bacterial plasmids containing the entire viral chromosome difficult, but methods for overlap recombination using restriction enzyme-digested adenoviral DNA and plasmids containing adenovirus sequence from the left end of the virus have been developed (1). Adenovirus variants in which restriction sites have been lost have been isolated (8) and are useful for overlap recombination. Alternatively, recombination can be accomplished by using a plasmid-based system in which a bacterial plasmid clone containing the entire adenovirus genome, which is too large to be packaged within the adenovirus capsid, is cotransfected with plasmids containing a left-end viral sequence (12). These systems work well but yield a fairly high background level of viruses arising from the parental viral DNA (13, 16). Further, the plasmid-based system employs a virus with partial deletion and replacement of E3 sequences (12), limiting the choice of vector backbones. This may present particular problems in instances in which the recombinant virus is to be injected into animals, since at least in the presence of E1A, the lack of E3 gene expression led to a great increase in the inflammatory response in cotton rats (3).

Distinction of the background plaques from overlap recombinants generally requires isolation and growth of the plaque followed by analysis of the DNA or product of the introduced gene. This approach presents a particular problem when the background level is high relative to the number of overlap recombination events. Therefore, a system in which initial selection can be made at the time of plaque isolation would be useful. We have developed adenovirus vectors suitable for overlap recombination which address these problems.

* Corresponding author. Mailing address: Department of Microbiology, Box B175, UCHSC, 4200 E. 9th Ave., Denver, CO 80262. Phone: (303) 270-6883. Fax: (303) 270-6785. Electronic mail address: schaack_j@defiance.hsc.colorado.edu.

Background virus resulting from ligation of adenovirus DNA arms during transfection. Ligation of transfected adenovirus fragments occurs with blunt ending, loss, or addition of sequence at the ends of the fragments (13). We have examined ligation events which occurred during attempts to make overlap-recombinant viruses. *dl*309 DNA was digested to completion with *Cla*I and *Xba*I, which cleave 919 and 1,340 bp, respectively, from the E1A end of the viral chromosome, and cotransfected with plasmid DNA containing the left end of the viral chromosome. Recombination between the large viral arm and the plasmid was expected to give rise to the desired virus. Viruses which had lost both the *Cla*I and *Xba*I sites but which did not appear to be the desired recombinants were isolated. Sequence analysis of the left-end DNA fragment demonstrated that the *Cla*I and *Xba*I sites were lost, apparently through filling in of the restriction sites and ligation of the ends in addition to insertion of 21 bp of DNA (Fig. 1). Examination of the inserted DNA indicated that it was identical to a segment near the origin of the bacterial plasmid vector. We have routinely observed a loss of restriction sites and the sequence between them after transfection of restriction enzyme-digested adenovirus DNA (data not shown). This evidence indicated that adenoviruses were arising from ligation events in the host cell during transfection leading to a significant background, as previously reported (13). This finding was used in designing viral vectors with which ligation events could be distinguished from overlap recombination.

Preparation of plasmids. The plasmid pXC15 (constructed by J. Logan and G. Winberg), which contains the left end of the adenovirus serotype 5 chromosome through the *Xho*I site at bp 5789, was modified by replacing the sequence between the *Sst*II site at bp 358 (this site delineates the 3′ border of the *cis*-acting packaging sequence [4, 5]) and the *Bgl*II site at bp 3329 (within the E1B coding region) with oligonucleotides containing *Bam*HI and *Bst*BI restriction sites. The *Escherichia coli* β-galactosidase gene driven by the cytomegalovirus (CMV) immediate-early promoter contained within a 1.1-kb

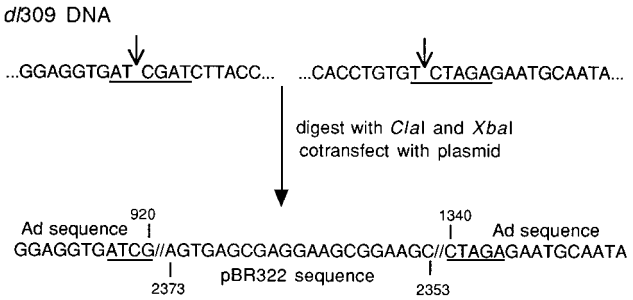

FIG. 1. Sequence replacement during overlap recombination. *dl*309 DNA (top sequence) was digested with *Xba*I and *Cla*I (the sites are underlined, and the positions of cleavage are indicated by arrows) and cotransfected with pXC15 DNA (containing the left end of the viral chromosome through the *Xho*I site at bp 5789) into which a nuclear matrix attachment site (10) had been inserted at position 107. DNA from a virus lacking the *Cla*I and *Xba*I sites was sequenced. The adenovirus (Ad) sequence surrounding the former *Cla*I and *Xba*I sites is indicated, with the bases remaining from the restriction sites underlined. The sequence inserted between the sites is completely homologous with the sequence between bp 2353 and 2373 of pBR322 (the vector backbone of pXC15).

fragment (a kind gift of K. Escudero, R. Smith, and C. Wilcox) was inserted into the *Bam*HI site of the modified pXC15. The resulting plasmid was designated pXC15β-gal (Fig. 2).

Selection and analysis of viruses containing the β-galactosidase gene in place of part of the E1 region of adenovirus. In order to obtain viruses which would offer simplification in isolation of subsequent recombinant viruses, the *E. coli* β-galactosidase gene was introduced into a variety of viral DNAs by using $Ca_3(PO_4)_2$ precipitates of pXC15β-gal and restriction enzyme-digested viral DNA (1) overlaid on 293 cells (6) (Fig. 2). The viruses used were strains *wt*300, which has a wild-type backbone and expresses the E3 products; *dl*327, in which the *Xba*I fragment between bp 28593 and 30471 within the E3 coding region is deleted so that a larger fragment of DNA can be incorporated into the viral chromosome; *dl*308 (9), which has *Xba*I sites at positions 1340 (within the E1A gene) and 10589 (at the 5′ border of the DNA encoding the main exon of the terminal protein) and therefore offers potential for the

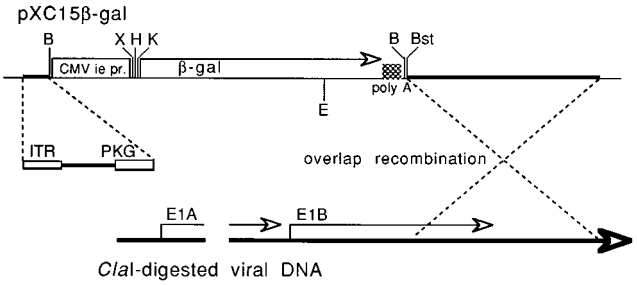

FIG. 2. Construction of adenovirus vectors containing the β-galactosidase gene. Viral DNAs (the left end of which is schematically represented by the horizontal line with the E1A and E1B genes indicated) were digested with *Cla*I (*dl*343 DNA was also digested with *Xba*I, which is not indicated). The restriction-digested viral DNAs were cotransfected with pXC15β-gal DNA, indicated by the bottom line. The β-galactosidase gene (β-gal) is schematically represented with the locations of the following restriction sites indicated: *Bam*HI (B), *Bst*BI (Bst), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), and *Xba*I (X). The locations of the CMV immediate-early promoter (ie pr.) and the polyadenylation site of simian virus 40 (checkered box) are indicated. The region of the plasmid encoding the left end of the viral chromosome is indicated in the enlarged region. ITR, inverted terminal repeat; PKG, *cis*-acting packaging element. The adenovirus sequence (from the left end through position 358 upstream and from position 3329 through 5789 downstream of the β-galactosidase gene) is indicated by the thick bar.

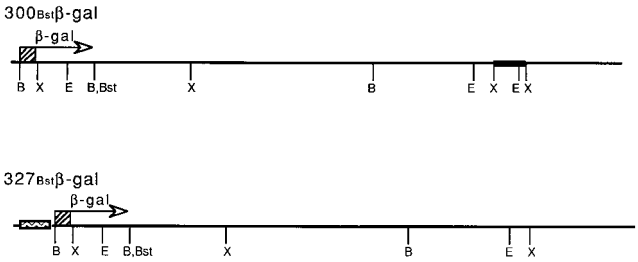

FIG. 3. Maps of β-galactosidase-expressing viruses. Restriction maps of 300_{Bst}β-gal (top line) and 327_{Bst}β-gal (bottom line) are presented schematically. The viral chromosomes are represented by the horizontal lines. The location of the β-galactosidase gene (β-gal) is indicated by the arrow, with the CMV immediate-early promoter fragment indicated by the boxes with diagonal lines. The additional sequence in 327_{Bst}β-gal which is not present in 300_{Bst}β-gal is indicated by the boxed area at the left. The *Xba*I restriction fragment between bp 28593 and 30471 (within the E3 region), including the *Eco*RI site at 30050, which is present in *wt*300 but not in *dl*327, is indicated by the thick line. The following restriction sites are indicated: *Bam*HI (B), *Bst*BI (Bst), *Eco*RI (E), and *Xba*I (X). In 327_{Bst}β-gal, the small *Xba*I fragment indicated by the thick line in 300_{Bst}β-gal has been deleted. 308_{Bst}β-gal (not shown) has an additional sequence introduced upstream of the β-galactosidase gene. The restriction maps are like those for the parental viruses except for the rearrangements, including the β-galactosidase gene, introduced at the left ends.

production of terminal-protein mutants; and *dl*343 (7), which has the *cis*-acting packaging sequence-E1A enhancer region reiterated at the right end of the viral chromosome and therefore has potential utility for introduction of a gene into the viral chromosome under the control of a specific promoter without a contribution from the E1A enhancer (since viruses in which the E1A enhancer is deleted from its normal location still package from the right end). Such viruses should permit color selection to be used as a first step in determining which virus plaques arise from recombinant viruses. A *Bst*BI site, a site which is not found in wild-type adenovirus serotype 5, was introduced downstream of the polyadenylation site for the β-galactosidase gene. The reasoning behind using a unique restriction site outside of the coding region for β-galactosidase was that if the ends of the viral arms were filled in and/or additional DNA was inserted between the restriction sites before ligation to yield a virus, the resultant virus would still yield a blue plaque in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

The restriction digestion patterns of 300_{Bst}β-gal, 308_{Bst}β-gal, and 327_{Bst}β-gal DNAs were examined by using *Hin*dIII, *Bam*HI, *Eco*RI, *Xba*I, and *Bst*BI. This analysis demonstrated that 300_{Bst}β-gal was configured as expected but that 308_{Bst}β-gal and 327_{Bst}β-gal contained additional sequences at the left end of the viral chromosome, upstream of the CMV immediate-early promoter–β-galactosidase cassette (Fig. 3). The additional DNA in the 308_{Bst}β-gal and 327_{Bst}β-gal chromosomes did not affect the ability of the viruses to function in overlap recombination (as discussed below). The exact nature of the extra DNA is being examined.

The β-galactosidase-expressing adenoviruses were found to grow to a 5- to 10-fold lower titer than the parental viruses in 293 cells (data not shown). The reduction in titer of the β-galactosidase-expressing viral stocks relative to that of the parental viruses may be due to the use of a 1.1-kb fragment of the CMV immediate-early promoter to drive expression of the β-galactosidase gene. Numerous viruses in which expression of a foreign gene driven by the CMV immediate-early promoter contained within a 760-bp fragment (lacking a sequence distal to the transcription start site) have been made. These viruses appear to grow to titers as high as those of the parental viruses.

Overlap recombination with adenovirus vectors expressing β-galactosidase. To test the ability of the recombinant β-galactosidase-expressing viruses to permit selection in introducing new genes into the adenovirus chromosome, a plasmid in which *Bst*BI sites were inserted after bp 107 and in place of the *Cla*I site at bp 919 within the E1A gene within the left-end plasmid pXC15, called $pXC15_{BstBst}$, was used for overlap recombination. The introduction of *Bst*BI sites on either side of the *cis*-acting packaging element is expected to lead to a virus useful for overlap recombination because of reductions in background. The background is due primarily to ligation of the viral arms (13). Elements which are required to make a viable virus include inverted terminal repeats at each end of the viral chromosome and a *cis*-acting packaging element near one end. By building viruses with restriction sites on either side of the *cis*-acting packaging element, it is expected that when these viral DNAs are used for overlap recombination, a three-fragment ligation will be required to yield a viable background virus and that the background will therefore be reduced. Five micrograms of $pXC15_{BstBst}$ was used in overlap recombination with 0.5 μg each of *Bst*BI-digested 300_{Bst}β-gal, $dl308_{Bst}$β-gal, and $dl327_{Bst}$β-gal DNAs ($dl343_{Bst}$β-gal was not used because it has the *cis*-acting packaging sequence reiterated at the right end of the chromosome; thus, a recombinant virus with *Bst*BI sites flanking the left-hand *cis*-acting packaging sequence would still be expected to require only that the viral ends, and not the internal fragment, be ligated together to yield a virus). Transfected and overlaid plates were stained with 100 μg of X-Gal per ml and 0.01% neutral red (Fig. 4). All of the clear plaques which were examined yielded the expected recombinant viruses, as analyzed by restriction digestion of the DNAs (Fig. 5).

Viral DNA from five plaques which were positive for β-galactosidase activity after transfection of *Bst*BI-digested $dl327_{Bst}$ β-gal viral DNA was examined for the presence of *Bst*BI sites. In all five viruses, the *Bst*BI site was lost, indicating that the original restriction digestion was efficient and that host cell activities had led to ligation of the viral arms after modification of their ends. Because the *Bst*BI restriction site is located outside the β-galactosidase gene, the loss of the site did not lead to a loss of β-galactosidase activity. Thus, background plaques behaved as predicted and this method of production of recombinants appears to allow efficient initial determination of recombinant plaques by simple staining.

300_{Bst}β-gal and $dl327_{Bst}$β-gal DNAs have been used in the construction of numerous other viruses. In the majority of these constructions, restriction digestion has indicated that the plaques which were clear after staining with X-Gal and which were tested were of the desired recombinant virus. In certain constructions, particularly those for which expression of the introduced gene appears to be deleterious to the virus, recombinant plaques have not always yielded the expected virus (13a). The new viral vectors have proved most useful in the construction of viruses in which a relatively high background level, which would normally entail significant effort in determining which plaques represent overlap recombination, has occurred.

We have found this system to work more efficiently than either a plasmid-based system (12) or the use of existent viruses (1, 8). The background in the plasmid-based system could potentially be reduced through the use of viral chromosomes lacking both the inverted terminal repeat and the *cis*-acting packaging sequence from the left end of the chromosome. Even with reduction in the background in other systems for overlap recombination, there would still be certain advantages inherent in the system described here. Among these are the facts that background events can generally be easily distinguished from overlap recombination by simple staining, permitting efficient selection of recombinant viruses; the β-galactosidase-expressing viral vectors grow to lower titers than do the parental viruses, offering a relative growth advantage to recombinant viruses; background plaques expressing β-galactosidase arise at a fairly high frequency, offering an internal control for the efficiency of both transfection and overlap recombination (thus, the use of unpurified viral arms actually offers certain advantages in this system); and the β-galactosidase-expressing viruses can be used as replication-defective controls for the effects of recombinant viruses expressing genes of interest.

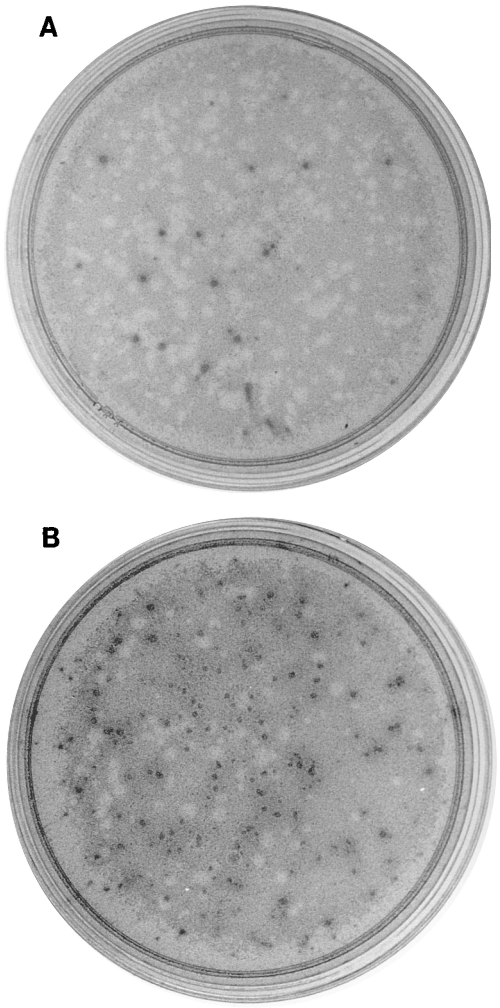

FIG. 4. Staining for recombinant plaques. 293 cells were cotransfected with *Bst*BI-digested 327_{Bst}β-gal DNA and pXC15 DNA. The monolayers were overlaid with 1% Noble agar containing medium and stained with neutral red (to distinguish viral plaques from living cells) and X-Gal (to distinguish background from overlap-recombinant plaques). β-Galactosidase-positive plaques are dark. The results of efficient overlap recombination (A) and the results of less-efficient overlap recombination (B) are shown.

Potential improvements to the system described here may be made through the use of promoters whose activity is downregulated in 293 cells, which should permit genes whose products are deleterious to viral growth to be incorporated into the viral genome, and incorporation of adenovirus mutations which lead to greater defectiveness in replication (for an ex-

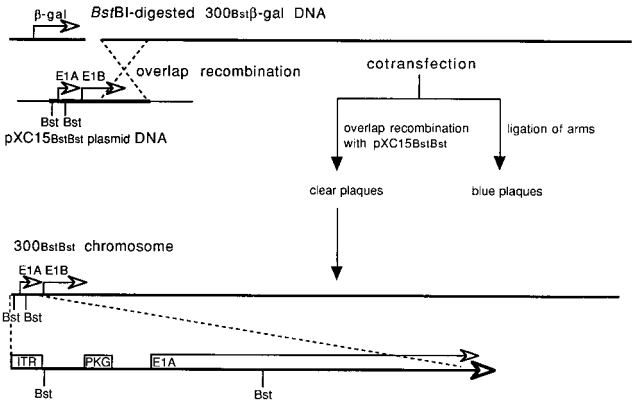

FIG. 5. Construction of viruses containing *Bst*BI sites flanking the *cis*-acting packaging element. *Bst*BI (Bst) sites flanking the *cis*-acting packaging sequence were introduced into *wt*300, *dl*308, and *dl*327 backgrounds by using the respective β-galactosidase-expressing vectors. The construction of *wt*300 with *Bst*BI sites is presented schematically. *Bst*BI-digested 300_{Bst}β-gal DNA, with the short, left arm (indicated by the shorter thick horizontal line) containing the β-galactosidase gene (β-gal; indicated by the arrow) and the long, right arm (indicated by the longer thick horizontal line), was cotransfected with the plasmid $pXC15_{BstBst}$ (the adenoviral sequence within $pXC15_{BstBst}$ is indicated by a thick line, with the locations of the E1A and E1B genes indicated by arrows and the bacterial plasmid vector indicated by thin lines) into 293 cells. Overlap recombination (indicated by crossed dashed lines) led to clear plaques, while ligation within the cell led to blue plaques. Viral DNAs from clear plaques were analyzed. 300_{BstBst} is schematically represented below 300_{Bst}β-gal, with the left end of the viral chromosome enlarged at the bottom. ITR, inverted terminal repeat; PKG, *cis*-acting packaging element.

ample, see reference 2), leading to an absolute lack of replication in the absence of appropriate complementation. With respect to the latter possibility, the isolation of 293 cell lines which express precursor terminal protein (14), a product essential for replication of adenovirus (reviewed in references 11 and 15), offers potential for the production of adenovirus vectors which are completely defective for replication.

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