

Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome

(virus vector/gene therapy/live vaccine)

SANAE MIYAKE*, MIHO MAKIMURA†, YUMI KANEGAE, SHIZUKO HARADA‡, YUMI SATO, KOICHI TAKAMORI, CHIKASHI TOKUDA§, AND IZUMU SAITO¶

Laboratory of Molecular Genetics, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, Japan

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ABSTRACT An efficient method of constructing recombinant adenoviruses (Ads) has been established. The expression unit to be introduced into recombinant Ad was first inserted into the unique *Swa* I site of the full-length Ad genome cloned in a cassette cosmid. The cassette bearing the expression unit was then cotransfected into human embryonic kidney 293 cells together with the Ad DNA-terminal protein complex digested at several sites with *Eco*T22I or *Ase* I/*Eco*RI. The use of the parent Ad DNA-terminal protein complex instead of the deproteinized Ad genome DNA allowed very efficient recovery of the desired recombinant Ad, and the above restriction digestion drastically reduced regeneration of the parent virus. Several hundred virus clones were readily obtained in each experiment, and about 70% of the clones were the desired recombinant viruses. Furthermore, because the cassette contained the full-length Ad genome, any position of the genome could be easily modified to develop a new vector design. We established construction systems for two types of Ad vectors, the E1-substitution type and the E4-insertion type. This method may greatly facilitate the application of recombinant Ads and should be useful for further improvement of Ad vectors.

The adenovirus (Ad) vector has attracted a great deal of attention as a vector for gene therapy (1, 2) and for possible live vaccines (3). It is also useful for studying gene functions in differentiated cells, such as neurons (4) and muscle cells (5). In spite of its usefulness, however, the extensive use of Ad vector has been hampered because the efficiency of obtaining the desired recombinant Ads by current methods has generally been poor.

The Ad genome is a 36-kb double-stranded DNA tagged with a 55-kDa terminal protein (TP) at both ends. When transfected to permissive cells, the Ad genome tagged with TP (Ad DNA-TP complex, Ad DNA-TPC) has been shown to produce 100-fold more viral plaques than normally prepared Ad DNA treated with proteinase (6). None of the current methods of constructing recombinant Ad have utilized Ad DNA-TPC. Saito *et al.* (7) previously attempted to utilize Ad DNA-TPC cleaved at two sites with a restriction enzyme instead of proteinase-treated Ad DNA, aiming to obtain recombinant Ad more efficiently. Although efficiency did increase, the attempt was unsuccessful because ultimately the vast majority of the virus clones obtained were parent virus derived from the DNA-TPC (7). Here we report that Ad DNA-TPC can be used successfully after DNA-TPC is cleaved at seven or more sites to abolish regeneration of the parent viruses; the efficiency of obtaining desired recombinant

Ads was improved almost 100-fold with no trouble caused by generation of parent viruses.

The Ad vector of the E1-substitution type has been the most popular to date and accepts an insert at a site near the left end of the Ad genome as a substitution for the Ad E1 region. On the other hand, the E4-insertion-type Ad vector (3, 7) accepts an insert at a site near the right end of the Ad genome, as an insertion upstream from the E4 region, with a compensatory deletion of the nonessential E3 region. The latter type of vector is particularly useful for constructing replication-competent recombinant Ads having the E1 region intact (3, 7). Such recombinant Ads have been considered candidates for live vaccines, possibly more effective than replication-deficient recombinant Ads. In this report we describe an efficient method for constructing both types of recombinant Ads utilizing cosmid cassettes and Ad DNA-TPC (COS/TPC method).

MATERIALS AND METHODS

Plasmid Construction. The cassette cosmid for constructing recombinant Ad of the E1-substitution type, pAdex1w (Fig. 1A), is an 11-kb charomid vector (8) bearing an Ad5 genome spanning (mu) 0–99.3 map units with deletions of E1 (mu 1.3–9.3) and E3 (mu 79.6–84.8). The unique *Swa* I site was created by linker insertion at the E1 deletion. The cassette cosmid for constructing recombinant Ad of the E4-insertion type, pAdex4w, similarly contained mu 2.6–100 of the Ad5 genome with the above E3 deletion. The Ad5 mu 76.1–100 sequence of the cosmid was derived from pX2S (7), and the unique *Swa* I site was introduced at the *Sal* I site, at mu 99.3, upstream from the E4 region. Two plasmids expressing *Escherichia coli lacZ*, pSR α lacZ (a generous gift from Y. Takebe, National Institute of Health, Japan) and pSRlacZ, were constructed by inserting the *lacZ* gene downstream from the SR α promoter of pcDSR α 296 (9). The initiation codons of the *lacZ* gene of pSR α lacZ and pSRlacZ were synthetic nucleotides, aagctt (*Hind*III)GC-ATG-Cct-gca g(*Pst* I) and ctgcag(*Pst* I)CAGACCGTGCATC-ATG-Agc ggc cgc(*Not* I), respectively. pAdex1SRw is an expression cosmid cassette derived from pAdex1w. The SR α expression unit was inserted at the

Abbreviations: Ad, adenovirus; TP, terminal protein; Ad DNA-TPC, adenovirus DNA-TP complex; mu, map unit(s).

*Present address: Department of Biochemistry, University of Toho, 5-21-16, Ohmorinishi, Ohta-ku, Tokyo 143, Japan.

†Present address: Department of Virology II, National Institute of Health, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162, Japan.

‡Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 75 Francis Street, Boston, MA 02115.

§Present address: Mitsui Pharmaceutical Industry Co., 1900-1, Togo, Mobara-shi, Chiba 297, Japan.

¶To whom reprint requests should be addressed.

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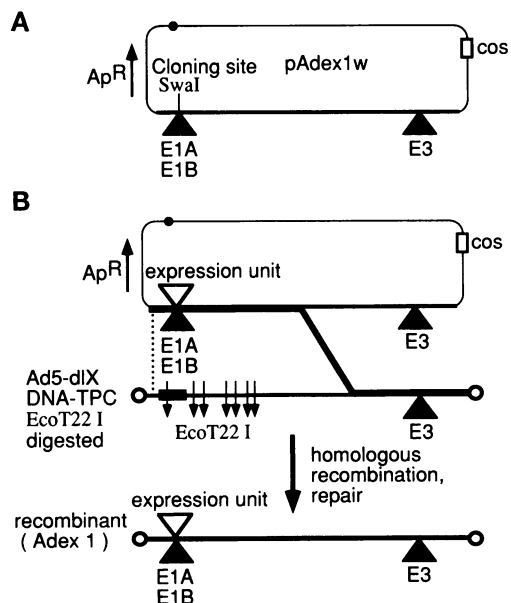


FIG. 1. Construction of recombinant Ads of the E1-substitution type. (A) The cassette cosmid pAdex1w. (B) The construction strategy. A cassette cosmid bearing an expression unit was cotransfected into 293 cells together with *EcoT22I*-digested DNA-TPC of Ad5-dlX. ApR, ampicillin-resistance gene; cos, *cos* site of λ phage; filled circles, pBR322 replication origin; open circles, Ad TP; vertical arrows on the Ad genome, restriction sites. The vertical dotted line represents a possible second homologous recombination.

deleted E1 region, and the cDNA cloning site just downstream of the promoter was converted to a *Swa* I site.

Insertion of an Expression Unit into the Cassette Cosmid.

An expression unit was excised with appropriate restriction enzymes, blunt-ended with the Klenow fragment of DNA polymerase I, and purified by gel electrophoresis. The fragment was mixed with 1 μ g of *Swa* I-linearized pAdex1w cassette DNA at a molar ratio of 3:1. After overnight ligation with T4 DNA ligase in a volume of 10 μ l, the DNA sample was digested with *Swa* I, and an aliquot (1/50th) was packaged *in vitro* using Gigapack XL (Stratagene). The *Swa* I cleavage site is at the center of the 8-nt sequence ATTT \downarrow AAAT, and hence *Swa* I digestion at this step cuts out the cassette/cassette junction that carries the regenerated recognition site for *Swa* I but does not cut at cassette/insert junctions that contain a new sequence, unless the insert happens to contain an *Swa* I site. Therefore, *Swa* I digestion before *in vitro* packaging served as a strong method of selecting cosmids containing the insert. Moreover, Gigapack XL prefers to package DNA exceeding the vector size of 42 kb and to select the cosmid containing the insert. In our hands, several thousand colonies were obtained after plating the transduced *E. coli* DH5 α , and the majority of the clones contained the desired insert. The same strategy was used for inserting cDNA into pAdex1SRw.

Construction of Recombinant Ad. Ad5-dlX has an E3 deletion (μ 79.6–84.8) (7), and Ad5-dl312 (10) has an E1A deletion (μ 1.2–3.7). These viruses were used as parent viruses for recombinant Ad construction. The DNA-TPC of the parent Ad was prepared through a buoyant CsCl density gradient with 4M guanidine hydrochloride (10). In brief, purified virions of the parent Ad purified through a buoyant CsCl gradient (11) were lysed by addition of an equal volume of 8 M guanidine hydrochloride, and the DNA-TPC released was purified through a buoyant density gradient of 2.8 M CsCl/4 M guanidine hydrochloride by centrifugation in a VTi65 rotor (Beckman) for 16 hr at 55,000 rpm. Then, DNA-TPC-containing fractions were identified by the ethidium bromide spot test, pooled, and dialyzed. The DNA-

TPC was then digested with *EcoT22I* (Takara Shuzo, Kyoto) or *Ase* I/*EcoRI* and was gel-filtered through a Sephadex G-50 spin column (12). The digested DNA-TPC was stable at -80°C . One microgram of the digested DNA-TPC was mixed with 8 μ g of cassette cosmid bearing the desired expression unit, and human embryonic kidney 293 cells in a 6-cm dish were transfected with the mixed DNA by the calcium phosphate method using a CellPfect Transfection kit (Pharmacia). 293 is a human embryonic kidney cell line transformed by Ad5 E1A and E1B genes (13) and supports propagation of E1-deleted recombinant Ads. The desired recombinant Ad was generated by overlapping recombination (14). One day later, the cells were spread in three 96-well plates at a 10-fold serial dilution mixed with untransfected 293 cells. After being maintained in culture for 10–15 days, virus clones were isolated and propagated further to assess restriction analysis and expression of inserted genes. The method used to propagate Ad was the standard procedure (11).

RESULTS

E1-Substitution-Type Vector System. To construct recombinant Ad containing an expression unit at the E1 deletion site, 1 μ g of Ad5-dlX DNA-TPC digested with *EcoT22I* was cotransfected into 293 cells together with 8 μ g of cassette cosmid pAdex1w bearing the desired expression unit at the *Swa* I site (Fig. 1B). *EcoT22I* cuts the Ad5-dlX genome at seven sites, all within the left one-third of the genome, thereby minimizing regeneration of the intact parent virus genome. The desired recombinant Ad was generated through homologous recombination occurring in 293 cells between the common regions of Ad5-dlX and the cosmid cassette from the rightmost *EcoT22I* site to the right end of the Ad5 genome (23.2 kb in length). It is known that the cosmid cassette sequences at the left end of Ad5 DNA are removed and repaired by utilizing the homologous inverted terminal repeat sequence intact at the left end of DNA-TPC (7, 15). However, removal and acquisition of the left-end TP could be efficiently achieved by the second homologous recombination at very near the left end of the Ad genome (i.e., after the insertion site; see below).

An average of 980 ± 360 virus clones were obtained in 15 construction experiments after establishment of this method. In each experiment, 6 viral clones were isolated and propagated further for extensive restriction analysis of the viral DNA. A typical result of the restriction analysis is shown in Fig. 2. Seventy \pm 15% of these clones in the 15 experiments were identified as the expected virus clones and only $12 \pm 11\%$ were the parent virus Ad5-dlX. The parent virus may have been generated by an accidental ligation of the leftmost two *EcoT22I* fragments of the parent virus genome with subsequent double homologous recombination between the Ad genome in the cosmid and both terminal fragments of the parent viral genome. The rest (18%) of the viral clones contained an incomplete expression unit of various lengths lacking its lefthand part. Such clones may have been generated through nonhomologous recombination connecting the *EcoT22I* end of the left-terminal Ad5-dlX fragment and any position within the expression unit. Therefore, the desired recombinant Ad was almost always obtained in a single experiment without any selection procedure. In our hands, the method was simple enough to allow one person to construct four different recombinant Ads simultaneously. Rearrangement or contamination of the parent virus had to be carefully investigated by restriction analysis after four serial passages. We found that the recombinant Ads generated were stable, the only exception being a particular construct containing a chimeric protein kinase C gene (see below). There was also one construct which we failed to obtain even after four trials. That was the only exception, however, and the expected recombi-

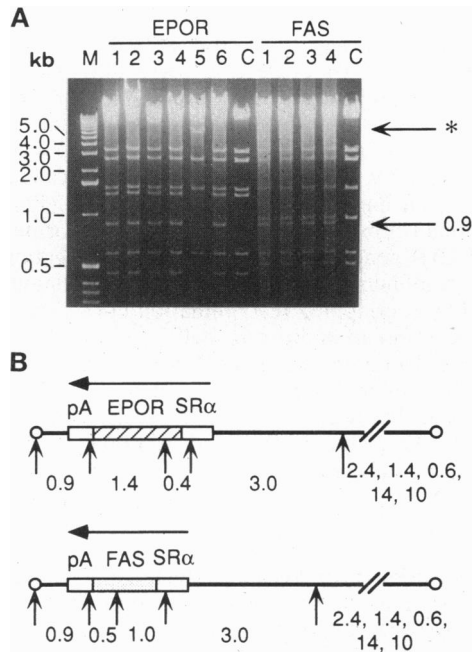


FIG. 2. Typical results of Ad clones generated by the COS/TPC method. (A) Total DNA samples from cells infected with Ad clones were digested with *Xho* I, subjected to agarose gel electrophoresis, and stained with ethidium bromide. The cDNAs of mouse erythropoietin receptor (EPOR) and mouse Fas antigen (FAS) were introduced into the Ad genome. Lane M, 1-kb ladder marker (GIBCO/BRL). All 10 clones except EPOR clone 5 show the restriction pattern of desired recombinant Ads. EPOR clone 5 shows the pattern of the parent virus, Ad5-dlX, and the arrow with an asterisk shows the 5.6-kb band covering the intact E1 region of Ad5-dlX (lane EPOR 5). The arrow with "0.9" indicates the 0.9-kb *Xho* I fragment specifically derived from the left terminus of the desired recombinant Ad genome. (B) *Xho* I restriction maps of the recombinant Ad genomes. SR α , SR α promoter; pA, polyadenylation sequence. Fragment sizes in kilobases are shown below the genome. Horizontal arrow indicates orientation of the transcription.

nant Ads have always been obtained thus far in the 50 different constructions of this type.

Construction of Adex4SRLacZL. Saito *et al.* (7) reported that an expression unit can be introduced into the Ad vector at a site upstream from E4 promoter (198 nt from the right end of the genome) (7). The Ad vector of this type (E4-insertion type) may be particularly useful for constructing replication-competent recombinant Ads with the E1A and E1B genes intact (3). We therefore next attempted to establish a system for constructing the E4-insertion type Ad vector utilizing Ad DNA-TPC.

As a model case, we chose the *lacZ* expression unit and E1A-deleted Ad5 (Ad5-dl312) (10). The Ad5-dl312 DNA-TPC digested with *Ase* I was cotransfected into 293 cells together with cosmid pAdex4SRLacZL, a derivative of pAdex4w containing the expression unit of pSR α lacZ at the site upstream of E4 in the left orientation (Fig. 3A). About 200 virus clones were obtained, a number comparable to that obtained with the E1-substitution system described above. However, only 4 clones out of 34 examined (11%) were found to be the recombinant Ad desired (Adex4SRLacZL). The remaining 30 clones had an unexpected structure, having acquired the E3 deletion from the cosmid without incorporating the *lacZ* expression unit (called Adex4B; see bottom of Fig. 3A). We carried out a similar experiment using an expression unit of the Ad12 E1A gene instead of the *lacZ* expression unit. The desired recombinant Ad, Adex4ATE1A, was found in only 4 clones of the 86 clones examined, with 5 clones of recombinants having an incomplete insert; the remaining clones

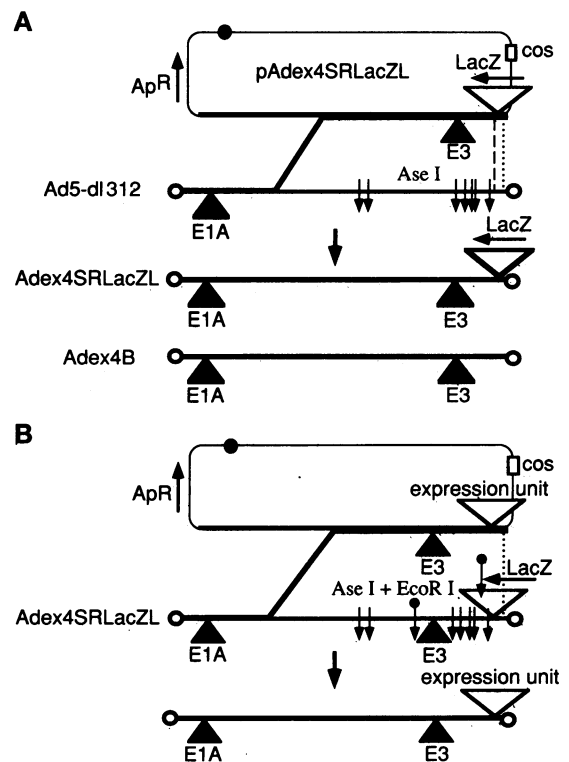


FIG. 3. Construction of recombinant Ads of the E4-substitution type. (A) Construction of Adex4SRLacZL. The vertical broken line represents a possible second homologous recombination before the *lacZ* insertion generating Adex4B, and the vertical dotted line represents a possible second homologous recombination after the *lacZ* insertion generating the desired recombinant Ad. Abbreviations are the same as in Fig. 1B. (B) Construction of recombinant Ads of the E4-insertion type. Adex4SRLacZL was used as a parent virus digested with *Ase* I plus *Eco*RI. Vertical arrow with filled circles represents the *Eco*RI site; arrow without circles represents the *Ase* I site.

[77 out of 86 (90%)] were again Adex4B. The majority of clones, Adex4B, were considered to have been generated through double recombination events (the expected recombination plus another one before the insertion site; see vertical broken line in Fig. 3A) as judged from their structure.

E4-Insertion-Type Vector System. There is an obvious difference between the two systems described above: the expression unit was introduced as a substitution for the E1 region of the parent virus in the E1-substitution system but as an insertion into the E4 gene of parent virus in the E4-insertion system. In the construction of the E1-substitution type, *Eco*T22I cuts once within the E1 region, and hence an additional recombination before the insertion site could not generate a full-length Ad genome. We therefore used Adex4SRLacZL as a parent virus instead of Ad5-dl312 to introduce the expression unit as a substitution for the *lacZ* unit of the parent virus (Fig. 3B). Then the core gene of hepatitis C virus under the control of SR α promoter, SR39, was used as a test, and the parent virus DNA-TPC was doubly digested with *Ase* I and *Eco*RI, the latter of which cuts the *lacZ* expression unit. All of the 30 resulted clones examined contained intact (14 clones) or partially deleted (16 clones) expression units, and no parent viruses or Adex4B were obtained. Similar successful results were obtained when Adex4ATE1A was used as a parent virus. This method was also successfully used to construct 6 more recombinant Ads [3 of them are described by Makimura *et al.* (16)]. Thus, we had established efficient and reliable methods for constructing Ad vectors not only of the E1-substitution type but also of the E4-insertion type.

Construction of Various Recombinant Ads. To conveniently construct recombinant Ad expressing the desired cDNA, we

then constructed an expression cassette cosmid, pAdex1SRw, containing the SR α promoter and poly(A) sequence. A given cDNA can be inserted into the unique *Swa* I site between the promoter and poly(A) sequence. The transcriptional orientation of the SR α promoter on the cassette is leftward, opposite to the original orientation of the E1A and E1B genes. We choose the leftward orientation because strong promoters such as EF1 α (17) and CAG (18) in the rightward orientation located at the E1-substitution site often yielded recombinant Ads of much lower titer than those containing the same expression unit with leftward orientation (data not shown).

We constructed four recombinant Ads of the E1-substitution type expressing *lacZ* in both orientations under the control of the SR α promoter (Adex1SRlacZL and Adex1SRlacZR) or EF1 α promoter (Adex1EFlacZL and Adex1EFlacZR). The recombinant Ads Adex1w and Adex1SRwL, which correspond to the cassettes pAdex1w and pAdex1SRwL without inserts, were also constructed. These recombinant Ads may be useful for many researchers. We also constructed recombinant Ads bearing the genes of hepatitis C virus C, E1, E2 proteins (19); hepatitis C virus NS3 protease (20), human interleukin 6 (21), human interleukin 6 receptor (22), human gp130 (IL-6 β -receptor) (23), human 4-hydroxyphenylpyruvate dioxygenase (24), mouse Fas antigen (25), mouse erythropoietin receptor (26), human ornithine transcarbamylase (27), phage P1 Cre recombinase (28), rat mak (male germ cell-associated kinase) (29), human 65-kDa neutrophil oxidase factor (30), mouse cAMP-dependent protein kinase (31), mouse cAMP-dependent protein kinase/bovine protein kinase C fusion protein (31), measles virus F protein (32), subacute sclerosing panencephalitis virus H protein (33), rat brain tau protein (34), human HST1 (35), promoterless G418-resistance gene (36), 5'-truncated G418-resistance gene (36), mouse granulocyte/macrophage-colony-stimulating factor (37), and human arylsulfatase A (38), as well as the *lacZ* gene under control of mouse myosin heavy-chain IIB promoter (39).

DISCUSSION

In this study an efficient and reliable method of constructing recombinant Ad was established. In our hands, construction efficiency is about 100-fold higher than that for the conventional method. The main reason for this high efficiency appears to be the use of Ad DNA-TPC instead of proteinase-treated Ad DNA. The previous attempt, however, failed to establish a practical method owing to the overwhelming generation of parent virus clones when the DNA-TPC was digested at two *Eco*RI sites before transfection (7). In the present method we employed *Eco*T22I and *Ase* I/*Eco*RI, both of which cut Ad DNA-TPC at seven or more sites. Such extensive fragmentation of DNA-TPC probably prevents the generation of the intact genome of the parent virus by the ligation which occurs in 293 cells and, hence, greatly reduces generation of the parent virus. The second reason for the high construction efficiency may be the increase in homologous lengths in the cassette and the parent Ad DNA, because the frequency of homologous recombination essential for generation of recombinant Ad is probably dependent on the above lengths. While the homologous length in the conventional system using the left-terminal *Xho* I fragment as a cassette was only 2.2 kb (μ 9.5–15.5), in the E1-substitution system in this study it was 10-fold longer (23.2 kb). The third reason could be that in our method some or most of the generated recombinant Ads acquire both TPs by double homologous recombination (the possible second recombination is indicated by a vertical dotted line in Figs. 1B and 3A and B).

The key material in the construction method is *Eco*T22I-digested Ad5-dIX DNA-TPC. Ad5-dIX, lacking only the E3 region, grows in HeLa cells as efficiently as the wild type. A 500-ml suspension culture of Ad5-dIX-infected HeLa cells yielded about 300 μ g of the DNA-TPC, sufficient to construct

300 or even 3000 recombinant Ads, if 1 or 0.1 μ g, respectively, of DNA-TPC is used for construction. Therefore, the digested DNA-TPC could be supplied as a ready-to-use kit for construction of recombinant Ad. This would greatly promote application of the Ad vector in many fields of basic biology and medical science. The E1-substitution-type vector theoretically has a cloning capacity of up to 7.5 kb, and in our experience a recombinant Ad with 6.8-kb insert could be obtained.

The method described here employs a cosmid cassette bearing nearly the full-length Ad genome. Usually cosmid cassettes are not preferred because a tedious linker ligation step is necessary to clone the insert at a very limited choice of cloning sites. We overcame this problem by employing *Swa* I as the cloning site, because (i) *Swa* I has no cleavage sites in the E3-deleted Ad5 genome; (ii) *Swa* I recognizes 8 nt, and hence it is very rare for an insert to happen to have the cleavage site; and (iii) the enzyme produces blunt ends. These features provide us with a very powerful method for selecting insert-containing clones by simply recutting before *in vitro* packaging. By using this procedure, together with a size-selective *in vitro* packaging system, a given DNA fragment excised with any combination of restriction enzymes can be cloned directly and very efficiently after a fill-in reaction. This feature also contributes much to wider use of Ad vectors through this method. Preparation of an even more improved version of the cassette cosmids with a polylinker containing the *Swa* I site (pAxcw and pAxCawt) has recently been achieved (40).

The cassette cosmid containing the full-length Ad5 genome is one of the best materials for improving Ad vector systems or studying Ad gene function by modifying Ad genes artificially. Bett *et al.* (41) developed a recombinant Ad construction system using a pair of plasmids sharing nearly the full length of the Ad5 genome, and recently, Ketner *et al.* (42) constructed a yeast artificial chromosome containing the full-length Ad2 genome, which was able to generate virus clones. However, the method described here offers not only easy manipulation of any position within the Ad genome but very efficient recovery of recombinant Ad, both of which are important for quick modification or development of new Ad vectors with a variety of new features.

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