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Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector

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ABSTRACT **Adenoviral vectors are widely used as highly efficient gene transfer vehicles in a variety of biological research strategies including human gene therapy. One of the limitations of the currently available adenoviral vector system is the presence of the majority of the viral genome in the vector, resulting in leaky expression of viral genes particularly at high multiplicity of infection and limited cloning capacity of exogenous sequences. As a first step to overcome this problem, we attempted to rescue a defective human adenovirus serotype 5 DNA, which had an essential region of the viral genome (L1, L2, VAI + II, pTP) deleted and replaced with an indicator gene. In the presence of wild-type adenovirus as a helper, this DNA was packaged and propagated as transducing viral particles. After several rounds of amplification, the titer of the recombinant virus reached at least 4 × 10^6 transducing particles per ml. The recombinant virus could be partially purified from the helper virus by CsCl equilibrium density-gradient centrifugation. The structure of the recombinant virus around the marker gene remained intact after serial propagation, while the pBR sequence inserted in the E1 region was deleted from the recombinant virus. Our results suggest that it should be possible to develop a helper-dependent adenoviral vector, which does not encode any viral proteins, as an alternative to the currently available adenoviral vector systems.**

For several reasons, adenoviruses are attracting increasing attention as expression vectors, especially for human gene therapy (1). First, the virion is relatively stable and can be prepared as a high titer stock ($\geq 10^9$ plaque-forming units/ml without purification). Second, adenoviruses can infect non-replicating cells. Finally, adenovirus vectors have been extensively exploited in vaccine development and proven safe in humans. The current adenovirus vectors have deletions in the E1 and/or E3 regions, because E1 proteins can be complemented in 293 cells, the E3 region is dispensable for growth of the virus in cultured cells, and all the other essential viral proteins are encoded in the vector itself. While the lower packaging limit of adenovirus is unknown, the upper packaging limit of adenovirus serotype 5 (Ad5) is ≈38 kb (2). As a result, vectors with deletions of E1 and E3 sequences (6 kb deleted in total) have a capacity for inserts of up to ≈8 kb (25). Because the sequences required in cis for replication and packaging of adenovirus DNA comprise <500 bp (3), it would be theoretically possible to accommodate up to 37 kb of insert DNA into defective adenoviral vectors by supplying all the proteins in trans from a helper virus or cell line. This development would permit delivery of multiple or large genes in one vector and delivery of all the elements of a gene needed in cis for properly regulated expression. Because such a vector would not encode any viral proteins that could be toxic or immunogenic to the host, the problem of the immune response of the host causing short-term expression from an adenoviral vector (4) might also be diminished. As a first step toward this goal, we report here the successful rescue and propagation of plasmid DNA encoding a defective adenovirus genome with a marker gene in the presence of a helper adenovirus.

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MATERIALS AND METHODS

Cell Lines and Virus. The 293 cell line (5), a human embryonic kidney cell line that constitutively produces E1 proteins, was maintained in minimum essential medium supplemented with heat-inactivated 10% newborn calf serum and 2 mM L-glutamine. The COS-7 monkey kidney cell line (American Type Culture Collection) was maintained in Dulbecco's modified minimum essential medium supplemented with heat-inactivated 10% newborn calf serum. Wild-type human adenovirus serotype 2 (Ad2) was obtained from ATCC. The Ad2 DNA/terminal protein (TP) complex was purified through a Sepharose CL-4B column (Pharmacia), dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA/2 mM 2-mercaptoethanol, and stored at 4°C as described (6).

Plasmid Construction. An expression cassette, SRαβ-geo, was constructed by subcloning the β-geo gene (7), a bifunctional fusion gene of *Escherichia coli* β-galactosidase (β-Gal) and neomycin resistance (*neo*) (kindly provided by P. Soriano), into the pCDLSRα296 plasmid (8) (kindly provided by Y. Takebe). The plasmid pKM74 was made by replacing the 6.9- and 0.4-kb *Sal* I fragments of pFG140 (9), a circular infectious genome of E1-defective human Ad5 with a plasmid insertion in E1A, with the SRαβ-geo expression cassette, resulting in a plasmid of ≈36 kb (Fig. 1).

Rescue and Propagation of the Vector. Five micrograms of pKM74 was cointroduced with 100 ng of Ad2/TP complex into 293 cells by a calcium phosphate transfection procedure (10) and the monolayer was overlaid with 0.5% agarose-containing medium (11). After plaques became visible, an additional overlay containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) at 0.01% final concentration was added to detect plaques expressing β-Gal. Blue plaques were picked and suspended in phosphate-buffered saline with 0.01% $CaCl_2$ and 0.01% $MgCl_2$ (PBS^{2+})/10% (vol/vol) glycerol, and the titer of the SRαβ-geo-containing virus was determined by infecting COS-7 cells followed by X-Gal staining (12). The blue-plaque isolates that yielded a higher number of blue cells on COS-7 cells were expanded by successive propagation on 293 cells in 24-well, 60-mm and 150-mm dishes.

Analysis of CsCl Gradient Fractions. After propagation in 293 cells cultured in 150-mm dishes, the virus stock was

Abbreviations: Ad2, adenovirus serotype 2; Ad5, adenovirus serotype 5; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-Gal, β-galactosidase; TP, terminal protein; ITR, inverted terminal repeat; moi, multiplicity of infection; SV40, simian virus 40.

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subjected to ultracentrifugation on a continuous CsCl gradient with a Beckman SW41 rotor (32,000 rpm for 18 hr at 4°C). The central part of the gradient, which contains a major band of the helper Ad2 and also possibly recombinant KM74 virus, was fractionated by collection from the bottom of the centrifuge tube. Each fraction corresponded to one drop of ≈50 μl. For DNA analysis, 2 μl of each fraction was diluted and incubated with 0.5 mg of proteinase K per ml in 10 mM Tris·HCl, pH 7.5/10 mM EDTA/0.5% SDS at 55°C overnight. DNA was prepared by phenol/chloroform extraction and ethanol precipitation and transferred to a nylon membrane (GeneScreen-*Plus*; NEN) using a slot blot apparatus (Minifold II; Schleicher & Schuell). The membrane was hybridized with Ad2 DNA as a probe by a standard procedure (13). After autoradiography, the intensity of the signal from each slot was quantified by using a laser densitometer (Ultroscan XL; LKB). Because the titer of the helper Ad2 was much higher than that of the recombinant KM74 virus as described below, this signal reflected the copy number of the helper Ad2 genome in each fraction. For analysis of the recombinant KM74 virus, 0.5 μl of each fraction was used to infect confluent COS-7 cells in 24-well dishes. After 16 hr, the cells were stained by X-Gal and the number of blue cells was counted by microscopic observation. To analyze the structure of the recombinant virus, three fractions that showed the highest β-Gal activity were pooled and dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA. Subsequently, DNA was purified, digested with restriction enzymes, separated by agarose gel electrophoresis, and subjected to Southern hybridization with SRαβ-geo and pBR probes by a standard procedure (13).

RESULTS

Construction of a Defective Adenovirus Plasmid. The pFG140 plasmid is an infectious circular genome of Ad5, which has an insertion of pMX2, a 2.2-kb derivative of pBR322 containing a plasmid replication origin and the ampicillin-resistance gene, at the *Xba* I site in the E1A region (9). After transfection into 293 cells, this DNA is replicated and packaged as linear molecules into viral particles to produce an E1-deficient adenovirus. The pKM74 plasmid is a derivative of pFG140 and has the SRαβ-geo marker gene, which replaces two *Sal* I fragments between map units 26.3 and 46.6 (Fig. 1). The total size of pKM74 is 35.7 kb. The coding region of L1

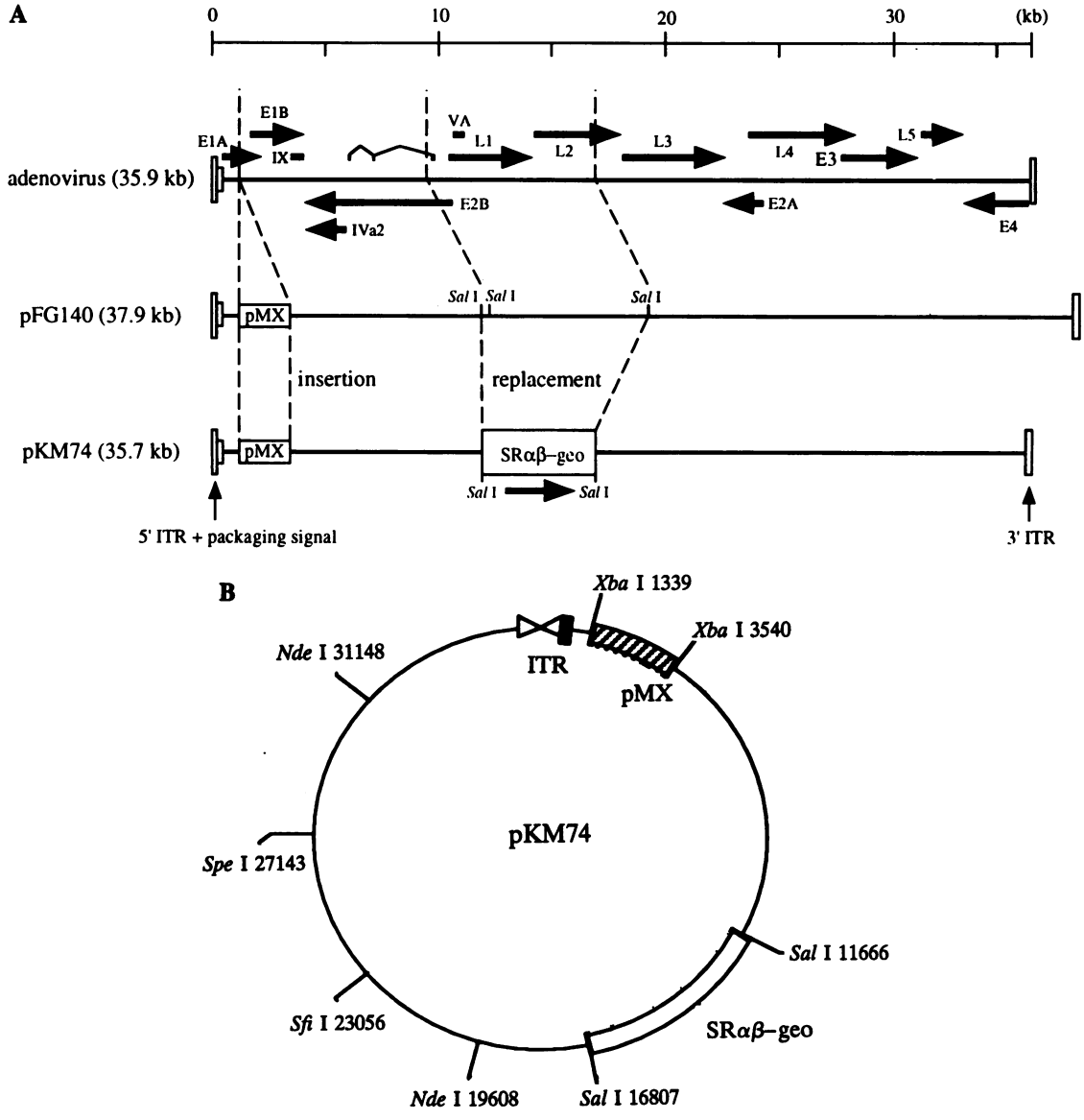

FIG. 1. Structure of pKM74. (*A*) pKM74 was made by replacing two *Sal* I fragments (corresponding to nt 9466–16750) of pFG140 with the SRαβ-geo cassette. The transcription map of adenoviruses is shown by thick arrows. (*B*) The two ITRs are covalently joined, as in pFG140, to form a circular plasmid.

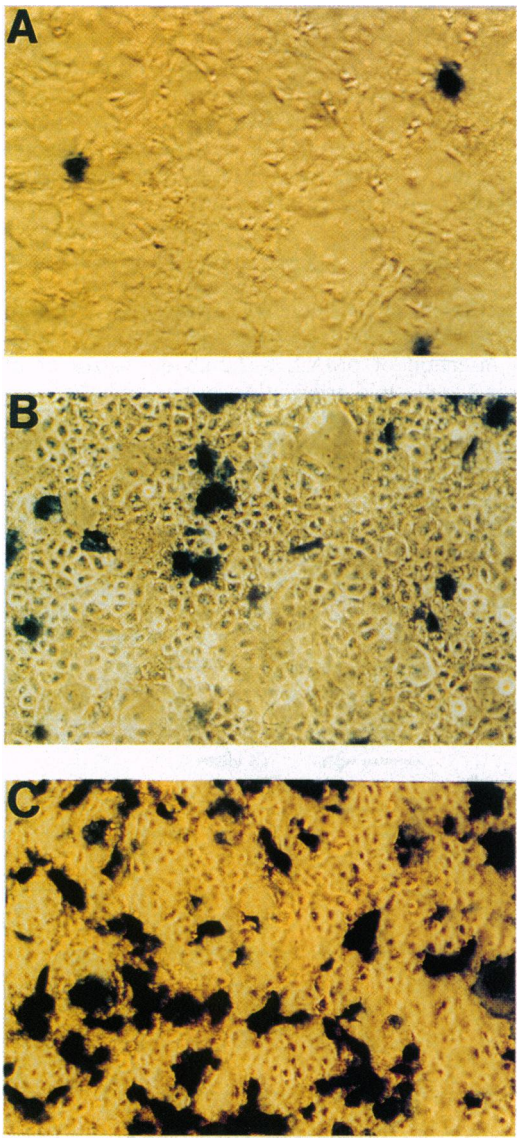

FIG. 2. X-Gal staining of COS-7 cells infected by KM74 virus no. 9. The virus was used to infect COS-7 cells in 24-well dishes. After 16 hr, the cells were fixed and stained with X-Gal. (*A*) Fifty percent of the primary plaque isolate. (*B*) Four percent of the isolate after the first passage on a 24-well dish of 293 cells. (*C*) One percent of fraction 16 after CsCl equilibrium centrifugation.

(encoding penton-associated protein), L2 (encoding core proteins), VAI and II, pTP, as well as the third late leader were partially or completely deleted in pKM74 (Fig. 1). As expected, transfection of this plasmid into 293 cells did not produce any progeny virus (data not shown). However, like the infectious plasmid pFG140, this pKM74 plasmid has the packaging signal, which is located between nt 194 and 358, and two nearly intact inverted terminal repeats (ITRs; nt 1–103), which act as a replication origin. pKM74 and Ad2/TP were mixed at a ratio of 50:1 and cotransfected into 293 cells. After plaques were formed, top agarose with X-Gal was overlaid to detect plaques containing pKM74 recombinant virus expressing β-Gal. About 1–5% of the plaques turned blue, suggesting that pKM74 was packaged and propagated as virus in these plaques (data not shown). To analyze the infectivity of the recombinant virus, several blue plaques were picked, suspended in PBS^{2+}/10% glycerol, and used to transduce COS-7 cells in 24-well dishes. A variable number of blue cells (0–400) were observed in different wells after infection with 50% of the virus suspension (Fig. 2*A*). No blue cells were observed when virus was heat inactivated at 60°C for 30 min (data not shown), suggesting that the expression of β-Gal was not caused by contaminating parental pKM74 plasmid DNA.

Propagation of Vector. The plaque isolates that showed strong β-Gal activity on COS-7 cells were propagated by serially infecting 293 cells in 24-well and 60-mm dishes. Increasing amounts of the KM74 virus were detected at each propagation step by using an aliquot of the infected cell extract to infect COS-7 cells for X-Gal staining, indicating that the KM74 virus was replicated (Fig. 2*B*). The appropriate amount of input virus was needed to obtain good amplification of the virus at each step. When the cells were infected at too high a multiplicity of infection (moi), the increase in KM74 virus titer was much smaller than that under optimal conditions. For example, 40 transducing viral particles of KM74 were amplified to 1.4×10^4 particles (350-fold increase) after one cycle of infection on one 60-mm dish, while 1000 particles were amplified to only 1.6×10^4 particles (16-fold increase) (Table 1).

Finally, two plaque isolates (isolate nos. 9 and 12) were used to infect 150-mm dishes of 293 cells. After 48 hr, the virus was extracted from the cell pellet by freezing and thawing and subjected to CsCl equilibrium density ultracentrifugation. The gradient was fractionated from the bottom of the centrifuge tube. To analyze the distribution of the helper Ad2 virus in the gradient, DNA from each fraction was subjected to slot blot hybridization with Ad2 DNA as a probe. To measure the distribution of the KM74 virus, each fraction was used to infect COS-7 cells for analysis by X-Gal staining (Fig. 2*C*). The summary of slot blot hybridization and X-Gal staining is shown in Fig. 3. In both isolates, the peak of helper Ad2 appeared between fractions 11 and 14, whereas the peak of KM74 virus measured by the X-Gal staining appeared at a lower density (fractions 16–18). Therefore, it seemed possible to partially separate the vector and helper virus by an equilibrium density-gradient centrifugation, although the sizes of the two viruses were similar at ≈36 kb. The titer of the helper virus at the peak (fraction 13) was $\approx 2 \times 10^9$ plaque-forming units per ml in both isolates. On the other hand, the titer of recombinants at each peak was 4.4×10^6 transducing virus per ml for isolate no. 9 and 1.2×10^7 transducing virus per ml for isolate no. 12. Considering a low sensitivity of the X-Gal staining, which requires 10^3 β-Gal molecules to detect a signal (12), the titer of the vector is likely to be underestimated. DNase I treatment of the virus did not abolish the β-Gal activity, indicating that the recombinant genome was packaged into virions (data not shown).

Structure of the Rescued Virus. The genome structure of the rescued KM74 virus no. 9 was analyzed by Southern hybridization after digestion of DNA with different restriction enzymes and agarose gel electrophoresis (Fig. 4). Because of the high sequence homology between the helper Ad2 and Ad5, which is the backbone of KM74, only the SRαβ-geo and pBR probes were informative. The restriction pattern of KM74 virus detected by the SRαβ-geo probe was identical to the expected pattern from the parental pKM74 plasmid with

Table 1. Amplification of KM74 virus by passage on 293 cells

Input virus	X-Gal-positive COS-7 cells	Total yield $\times 10^{-4}$	Increase, -fold
40	140	1.4	350
100	100	1.0	100
400	60	0.6	15
1000	160	1.6	16

Different amounts of KM74 virus were used to infect one 60-mm dish of 293 cells. After 48 hr, the cell pellet was suspended in 1 ml of PBS^+/10% glycerol. Titer of the amplified stock was determined by infecting COS-7 cells with 10 μl of the extract, followed by X-Gal staining.

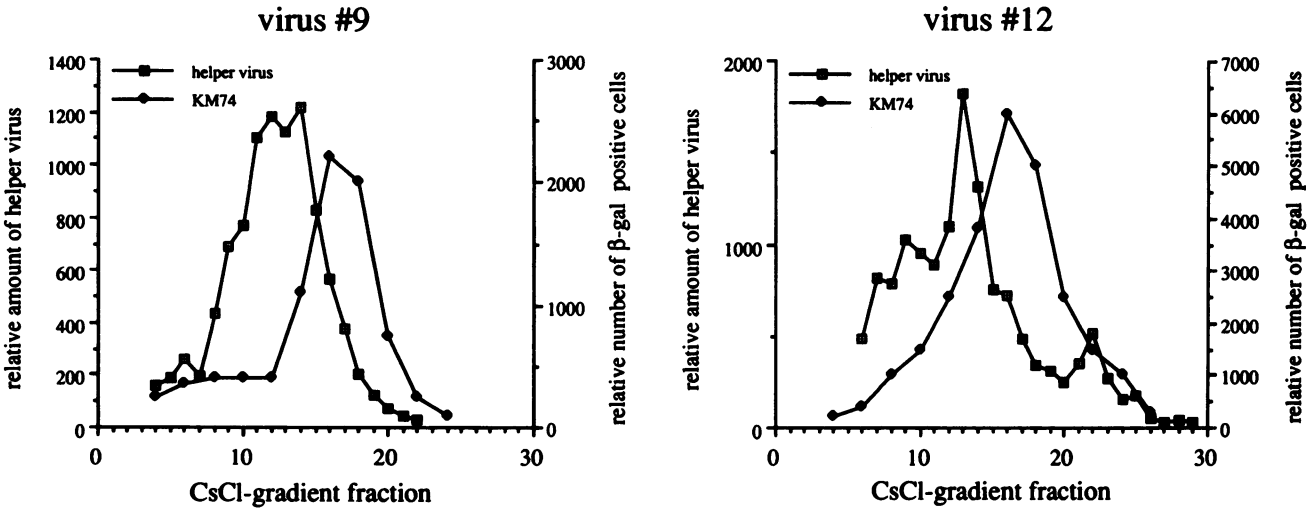

FIG. 3. Fractions from the CsCl gradient of KM74/Ad2 viruses 9 and 12. Fractions were collected from the bottom (lower number) to the top (higher number) of the gradient. Viral DNA was extracted from individual fractions and subjected to slot blot hybridization. Relative intensity of the signal using Ad2 DNA as a probe, corresponding to the copy number of the helper Ad2, is shown as open squares. Number of blue cells after infecting COS-7 cells with 0.5 μl of each fraction, corresponding to the number of KM74 virions, is shown as solid diamonds.

*Hin*dIII, *Sac* II, and *Xba* I (Fig. 4). When the same blot was hybridized with the pBR probe, no signal was obtained (data not shown), suggesting that the pMX2 sequence was deleted from the KM74 virus. The same result was obtained from virus 12 (data not shown). This may have been the result of an early recombination event between Ad2 and KM74 sequences in the left part of the viral genome. However, because of the lack of appropriate restriction sites distinguishing Ad2 and Ad5, it was impossible to determine where a recombination or deletion event had taken place on the KM74 virus.

DISCUSSION

Recombinant adenoviral vectors have been shown to be potential new tools for a variety of human gene therapy protocols. The currently available adenoviral vectors (typically, E1 substitution mutants with or without deletion of E3) are propagated on 293 cells, which provide the essential E1 proteins. Because no other viral functions are provided by this cell line, the vectors have to encode other essential viral genes, resulting in a limited cloning capacity of foreign DNA (≈8 kb) (25). The presence of a large DNA segment that encodes various viral proteins might be the main reason for an immune response against the virus by the host (4). As a first step to overcome these limitations, we attempted to rescue a defective adenovirus vector DNA by providing essential viral functions from a helper virus. The vector has a 7.23-kb deletion in an essential part of the genome carrying L1, L2, VA, and TP, with the SRαβ-geo marker gene as a substitution, and an insertion of pMX2, a pBR322 derivative, in the E1A gene. Therefore, this plasmid could not make infectious progeny after transfection into 293 cells, although some of the viral proteins still might be

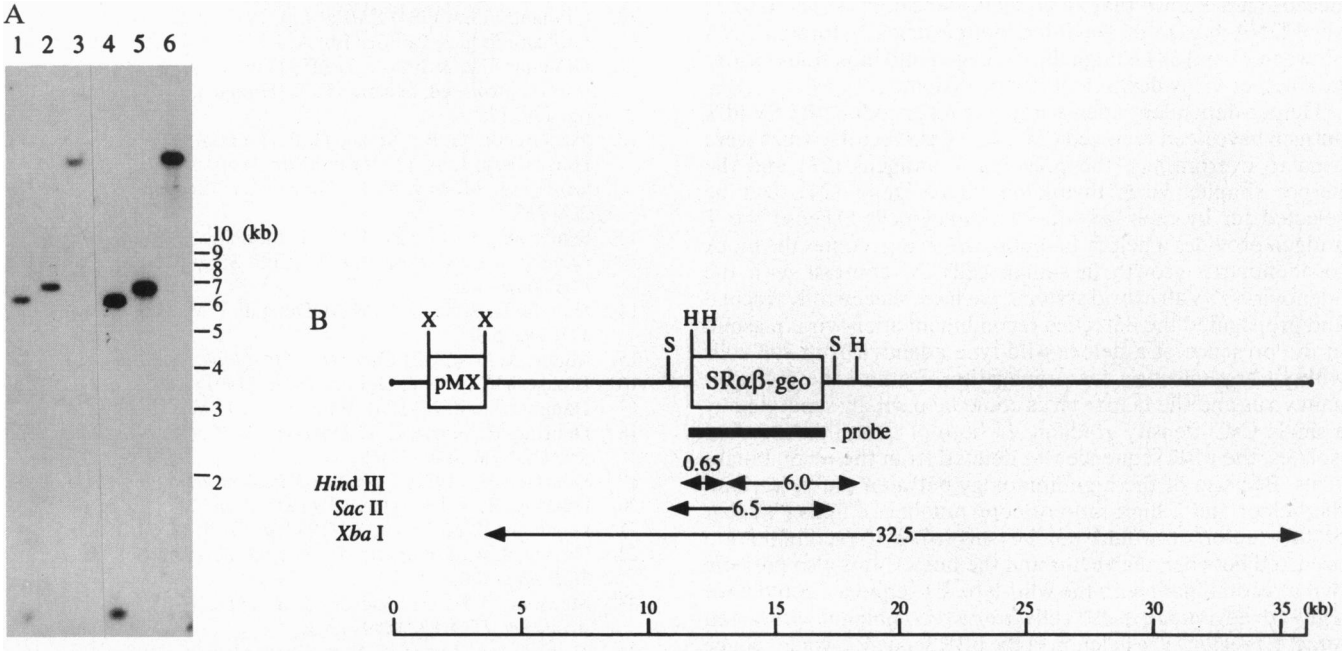

FIG. 4. Analysis of the structure of the KM74 virus. (*A*) Viral DNA was extracted from pooled fractions, digested with different enzymes, and subjected to Southern hybridization. The SRαβ-geo fragment was used as a probe. Lanes: 1–3, KM74 virus; 4–6, parental pKM74 plasmid; 1 and 4, *Hin*dIII; 2 and 5, *Sac* II; 3 and 6, *Xba* I. (*B*) Restriction map of KM74 genome. Only the restriction sites in the 5′ half of the genome, which hybridizes to the SRαβ-geo probe, are shown here. Position of the probe is indicated by a thick bar.

expressed. The size of this plasmid, pKM74, is approximately the same as that of a wild-type adenovirus (Fig. 1). Because this plasmid contains the packaging signal (3), located at the 5′ end of the genome, we hypothesized that after replication as a viral genome (9), the plasmid DNA as well as the genome of helper adenovirus should be encapsidated as a linear molecule in adenovirus virions. The replication origins in the ITRs should allow this plasmid to be replicated in the transduced cells as a result of expression of Ad2 proteins including viral DNA polymerase, DNA binding protein, and pTP supplied by the helper Ad2 (14). Because the plasmid itself is replication incompetent, this system is similar to retroviral vectors (15) or herpes simplex virus type 1 amplicon vectors (16).

Because all the cis elements for packaging and replication are contained in only 360 bp from the left end of the genome and 103 bp from the right end, we propose that it will be feasible to delete most of the viral genome in a helper virus-dependent system and to enrich the recombinant virus by repeated fractionation on CsCl gradients and large-scale infection of 293 cells with the recombinant virus-containing fractions. This hypothesis is supported by previous observations of other groups. First, it was documented that after repeated passaging at high moi on permissive cells with different adenovirus serotypes, subgenomic DNA preferentially containing the left end of the virus genome could be packaged into virions and separated from wild-type virions by CsCl density-gradient centrifugation (17). Second, after repeated high moi passage of Ad12 on human KB cells, hybrid viruses containing symmetrically duplicated chromosomal DNA of KB cell line flanked by 700–1150 bp of DNA fragment from the left terminus of Ad12 (SYREC) were produced (18–20). These SYREC could be separated from Ad12 by a CsCl equilibrium density gradient and also could be propagated over years together with Ad12. Third, hybrid viruses between simian virus 40 (SV40) and Ad5 have also been reported. One of the viruses comprised only 3.5 kb of DNA from the left end of Ad5 and 5.5 copies of SV40 DNA with a total size of 35 kb (21). In addition, the smallest genome size among different types of Ad5/SV40 chimeric viruses was ≈25 kb (22). These reports suggest that there might be a lower size limit of the adenovirus genome to be propagated in virions. Because it is known that an adenovirion can package up to 38 kb of DNA as a stable and infectious particle (2), foreign DNA between 25 and 38 kb might be packaged into infectious virions in a helper virus-dependent vector system.

Helper-dependent adenoviral vectors encoding the SV40 T antigen have been reported (23, 24). These vectors, which were used to overproduce the polyoma T antigens (23) and the herpes simplex virus thymidine kinase gene (24), can be selected for by their growth in monkey cells because the T antigen provides a helper function, which overcomes the block to adenovirus growth in simian cells. In contrast with the adenovirus/SV40 hybrid systems, we have successfully rescued and propagated the defective recombinant adenoviral plasmid in the presence of a helper wild-type adenovirus in 293 cells without any selection for propagation. Rather, the recombinant virus and the helper virus could be partially separated by a single CsCl density gradient. In both of two different virus isolates, the pBR sequence was deleted from the recombinant virus. Because of the high homology between the vector and the helper and a high ratio of copy number of helper to that of the vector, it would not be surprising if recombination occurred between the vector and the helper. It is also possible that a recombinant with the wild-type E1 sequence could have a growth advantage in 293 cells over a recombinant without an intact E1 region. The deletion of the pBR sequence would reduce the size of the recombinant virus genome, and this could explain why the recombinant virus appeared at a slightly lighter density than the wild-type Ad2 helper on a CsCl gradient.

In summary, we have shown that it is possible to rescue and propagate a defective adenoviral genome with a gene of interest in the presence of a helper virus. With appropriate design, the problem of recombination between vector and helper virus, as was observed in this study, should be minimized. For human gene therapy, it will also be important to examine whether an E1-defective virus can be used as the helper in this system. By improving vector design and using a better helper virus it might be possible to develop a safe adenoviral vector system with a large cloning capacity. For example, by increasing the size difference between the vector and the helper virus, a more satisfying separation between the vector and the helper virus might be achievable. While the current system is not directly applicable for human gene therapy, it will provide the means to test immunogenicity and leaky gene expression in truncated adenoviral vectors. This would be of considerable value for human gene therapy as well as other *in vivo* and *in vitro* gene transfer experiments.

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