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A Helper-Dependent System for Adenovirus Vector Production Helps Define a Lower Limit for Efficient DNA Packaging

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Adenoviruses (Ads) are intermediate-sized mammalian DNA viruses with a double-stranded linear genome of 36 kb. The icosohedral virion has been shown to accommodate up to 105% of the wild-type genome length, and genomes larger than this size are either unpackageable or extremely unstable, frequently undergoing DNA rearrangement. Here we show that the Ad virion also has a lower packaging limit of approximately 75% of the wild-type genome length. We have constructed a series of vectors with sizes ranging from 15.1 to 33.6 kb and used these to show that in our Cre/*loxP* helper-dependent system (R. J. Parks, L. Chen, M. Anton, U. Sankar, M. A. Rudnicki, and F. L. Graham, Proc. Natl. Acad. Sci. USA 93:13565–13570, 1996), vectors with genomes greater than or equal to 27.7 kb are packaged with equal efficiencies, whereas vectors with smaller genomes are inefficiently packaged. A 15.1-kb vector, approximately half the size of the wild-type adenovirus genome, was packaged with an efficiency intermediate between that of the small (21.3- to 25.7-kb) and large (27.7- to 33.5-kb) vectors. Analysis of vector DNA after amplification in helper virus-infected cells showed that vectors below 75% of the Ad genome had undergone DNA rearrangements, whereas larger vectors were unaltered. The 15.1-kb vector was recovered primarily as a mix of head-to-tail and tail-to-tail covalent dimers, with a size of 30 kb. We conclude that the Ad virion can efficiently accommodate viral DNA of greater than 75% of the viral genome but that smaller viral genomes tend to undergo rearrangement, resulting in a final size of greater than ~27 kb before they can be efficiently packaged. Knowledge of the lower limit to Ad DNA packaging should allow for the design of better and more stable vectors.

Adenoviruses (Ads) are a family of DNA viruses characterized by icosohedral, nonenveloped capsids containing a linear double-stranded DNA genome (21). The human Ad type 5 (Ad5) is approximately 36 kb and encodes genes that are divided into early and late viral functions. Ad5 can be rendered replication defective by removal of the E1 region, which is located at the left end of the genome and is involved in transactivation of other viral early and late genes (E1A functions), in regulation of host cell and viral RNA and protein synthesis, and in protecting the cell from E1A-induced apoptosis (E1B functions). Thus, E1 functions are essential for viral replication. E1-deleted virus can be propagated in 293 cells, which express E1 of Ad5 (19), and such viruses have been used for the introduction and expression of foreign genes in mammalian cells for gene therapy, as recombinant viral vaccines, or as general-purpose expression vectors for experimental studies (3, 18). E1-deleted (first-generation) Ad vectors have a cloning capacity of ~4.7 kb of foreign DNA (up to 105% of the wild-type genome) (4). Deletions in the nonessential E3 region can further increase the cloning capacity to ~8 kb. Ad vectors with DNA inserts that result in genomes larger than 105% of the wild-type DNA are, however, nonviable or unstable and frequently undergo DNA rearrangements to reduce the overall size of the vector DNA (4). Nevertheless, currently available vectors permit the cloning and expression of most cDNAs and can be used to infect a wide variety of both replicating and nonreplicating cells (6, 7). The replication-defective nature of these vectors, their relatively large cloning capacity, and the ease of genetic manipulation and preparation of high-titer stocks have fuelled considerable interest in Ad-based vectors for gene therapy.

Although first-generation, E1-deleted vectors are replication defective in nonpermissive (i.e., $E1^-$) cells, several studies have indicated that under certain conditions, leaky expression of viral genes can occur in transduced cells (10, 12, 15, 30, 31). In vivo, this may contribute to the induction of an immune response against the virus or transduced cells, resulting in only transient transgene expression and in reduced effectiveness of subsequent vector readministration (10, 14). Therefore, numerous attempts have been made to develop new vectors in which additional viral genes, besides those encoded by E1 or E3, are deleted or attenuated (1, 2, 11, 16, 23, 27, 32), and these vectors are associated with a reduced immunogenicity in vivo (11, 15, 29).

Recently, we and others have developed helper-dependent systems for the generation of vectors that lack all coding sequences of the Ad genome (13, 22, 24–26). In these systems, a helper virus provides in *trans* all of the proteins required for the packaging of the vector, which contains only those *cis*-acting elements required for viral DNA replication and packaging, mainly the inverted terminal repeat sequences and packaging signal. Since these latter elements are contained within ~500 bp from the ends of the genome (17), helper-dependent vectors have the potential to range in size from a few hundred base pairs to slightly greater than the size of wild-type Ad and to carry up to ~37 kb of foreign DNA. However, vectors that have substantially less DNA than wild-type Ads undergo rearrangements and multimerizations (13, 20), resulting in a net increase in vector size. This suggests that there may be a lower limit to the amount of DNA that is efficiently packaged into virions. Using our Cre/*loxP* helper-dependent system (26), we show that Ad virions preferentially package DNA of greater than ~75% of the wildtype genome and vectors smaller than

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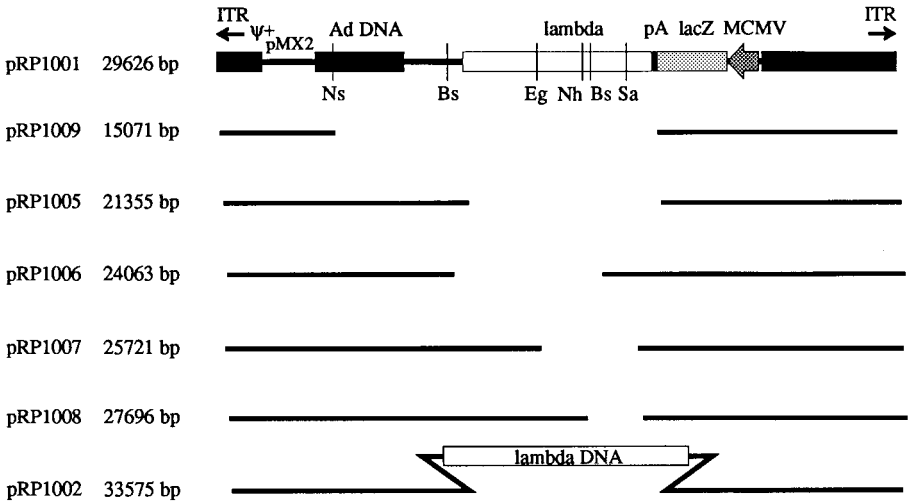

FIG. 1. Vectors used in the helper-dependent system. pRP1001 is a derivative of pFG140 (17a) and retains Ad sequences corresponding to 5,789 bp of the left end, including the viral packaging signal (Ψ+), and 6,143 bp of the right end of Ad5 (26). The E1A-coding region is disrupted by the insertion of pMX2 (which contains a bacterial origin of replication and ampicillin resistance gene) at the *Xba*I site (1,338 bp). pRP1001 also encodes a β-galactosidase (*lacZ*) reporter gene under the regulation of the murine cytomegalovirus immediate-early promoter and simian virus 40 polyadenylation signal and a lambda DNA stuffer sequence. Other vectors were constructed by complete or partial digestion with appropriate restriction endonucleases, repair with T4 DNA polymerase, and recircularization of the plasmid with T4 DNA ligase, using standard techniques (26a). pRP1002 contains a large insert of lambda DNA (corresponding to 19,400 to 31,620 bp of the conventional lambda map), replacing the lambda sequences in pRP1001 and yielding a vector of 33,575 bp.

this appear to be packaged with a much lower efficiency. Furthermore, vectors smaller than 27 kb are unstable and undergo DNA rearrangements, resulting in a net increase in the size of the vector to greater than 27 kb.

The Cre/*loxP* helper-dependent system. We have previously described a helper-dependent system that uses a helper virus with a *loxP*-flanked packaging signal (26). On infection of a 293-derived cell line that constitutively expresses the Cre protein (8), the viral packaging signal is efficiently excised via Cre-mediated site-specific recombination between direct repeats of *loxP*, rendering the virus unpackageable. However, the viral DNA is able to replicate and express all the proteins required in *trans* to package an Ad vector encoding the appropriate *cis*-acting elements. Plasmids containing the viral packaging signal (Ψ) and inverted terminal repeats (ITRs) joined head-to-tail are transfected into cells infected with the helper virus, in which they are replicated into linear genomes that are then packaged into infectious virions. The titer of the vector can be increased by serial passage through helper virus-infected 293Cre cells. This system has the advantage that only ~500 bp of Ad-specific sequences are required in the vector and, thus, a wide range of sizes of foreign DNA can be accommodated. Furthermore, because the helper virus genome is prevented from packaging after Cre-mediated excision of Ψ, the vector can be efficiently replicated and prepared nearly free of contaminating helper virus.

Effect of vector DNA size on efficiency of vector recovery. To examine the effect of vector DNA size on packaging efficiency, we constructed a series of plasmids ranging in size from 15.1 to 33.6 kb, containing Ψ and the ITRs, as well as a β-galactosidase (*lacZ*) reporter gene under the control of the murine cytomegalovirus immediate-early promoter and simian virus 40 polyadenylation signal (Fig. 1). 293Cre4 cells were transfected with these plasmids and infected with AdLC8cluc helper virus (26) at a multiplicity of infection (MOI) of 5 PFU per cell. After complete cytopathic effect (CPE) (~48 h postinfection), the cells were harvested and freeze-thawed to release the virus. Aliquots (500 μl per 60-mm-diameter dish) of the crude viral lysates were used to infect 293 monolayers, which were stained 20 h later to detect β-galactosidase expression. No blue-staining cells were detected in cultures that were infected with control extracts prepared from transfection/infections lacking either AdLC8cluc or vector DNA. Plasmids that were larger than 27 kb were efficiently converted into packageable linear molecules, as shown by the recovery of β-galactosidase-transducing particles (blue-forming units [BFU]) (Fig. 2A). Vectors of less than 26 kb were recovered at a significantly reduced frequency, with an average recovery of less than half that of the larger vectors (175 versus 452 BFU per pmol of transfected DNA). Curiously, AdRP1009, which is approximately half the size of the wild-type Ad genome, was packaged at a higher efficiency than the other small vectors, which ranged in size from 21.3 to 25.7 kb.

Inefficient recovery of BFU after calcium phosphate-mediated DNA transfection could be due to other factors besides inefficient packaging, such as inefficient conversion to linear replicating DNA molecules. Therefore, we examined further the effect of vector length on packaging efficiency by subjecting the viral lysates from the initial transfection to serial passage through AdLC8cluc-infected 293Cre4 cells. For the first passage, 293Cre4 cells were infected with 500 μl of vector-containing lysate. No additional helper virus was added since AdLC8cluc can replicate in the 293 cells used in the initial transfection and was present in large quantities in the vector-containing lysate. After the first round of amplification, we examined the burst size for each virus. Virus input was calculated based on the titer of the vector after the initial transfection. The output of virus was the total amount of vector obtained after the first amplification in 293Cre4 cells, and the burst size was calculated as the total viral yield divided by the virus input. Vectors with an overall size of between approximately 21 and 26 kb yielded a burst size ranging from 9 to 30, whereas vectors of greater than 27 kb had a burst size ranging from 280 to 329, or an average of 17 times higher than that observed for the smaller vectors (Fig. 2B). AdRP1009 (15.1 kb) continued to be more efficient than the other small vectors and had a burst size of 95, or approximately 3- to 10-times that of the other vectors less than 27 kb.

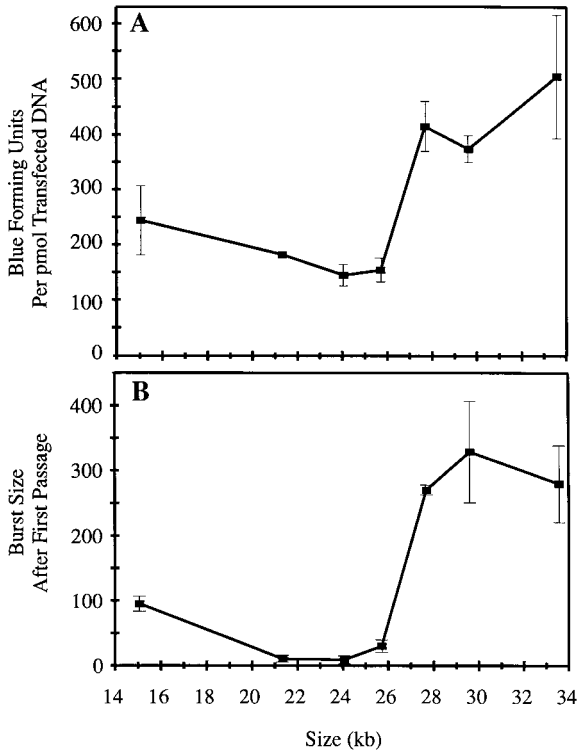

FIG. 2. Rescue and amplification of helper-dependent vectors. The methods for transfection, amplification, and analysis of our helper-dependent vectors have been described previously (26). Briefly, plasmid DNAs (5 μg per 60-mm-diameter dish) were transfected into 293 cells and the cells were infected 18 h later with AdLC8cluc at an MOI of 5. The cells were incubated until the monolayer showed complete CPE; they were then harvested, and the virus was released by three rounds of freezing and thawing. (A) An aliquot of the resulting crude viral lysate (500 μl) was used to infect 293Cre4 cells for assays of *lacZ*-expressing virus, and the results were expressed as BFU per pmol of transfected DNA. Two independent transfection/infections were performed and graphed as an average, with error bars representing high and low values. (B) Aliquots (500 μl) of the crude vector lysates from the initial transfection were used to infect 293Cre4 cells. After complete CPE, the crude vector lysates were assayed for the presence of BFU. The burst size was calculated as the total vector yield after the first amplification divided by the vector input (as BFU). The data are expressed as an average of two independent transfection/infections, and error bars represent the high and low values.

Serial amplification of vectors. We next subjected the different vectors to additional serial passage in AdLC8cluc-infected 293Cre4 cells and measured their titers (BFU) after each passage. Similar quantities of each vector were used as inoculum for the first passage. Each amplification of AdRP1008, AdRP1001, and AdRP1002 (27.7 to 33.6 kb) resulted in a 10- to 100-fold increase in virus titer (Fig. 3), but recovery of vectors of less than 26 kb was reduced over all passages. Again, AdRP1009, which is approximately half the wild-type Ad5 genome length, was amplified at a rate that was intermediate between that of other vectors of greater than or less than 27 kb in size. We conclude that vector DNA of less than ~75% of the wild-type genome was replicated and/or packaged inefficiently and vectors greater than this size are packaged with equal efficiency.

Cesium chloride buoyant density and Southern analysis of AdRP1001, AdRP1005, and AdRP1009. Our observation that a vector approximately half the size of the wild-type Ad genome is replicated with a higher efficiency than other small vectors can be explained in two ways. It is possible that the packaged vector is a covalent dimer of the transfected plasmid, which results in a molecule of ~30 kb, a size which should be very efficiently packaged. Packaging of multimerized DNAs has been observed in Epstein-Barr virus (5) and Ad (13). Alternatively, the virion may contain two monomer copies of the vector, again increasing the size of the encapsidated DNA to 30 kb. The latter is not an unlikely possibility since packaging of multiple DNAs into a single phage head has recently been reported in bacteriophage P1 (9). Either of these mechanisms would result in the incorporation into the virion of two copies of a foreign gene encoded by the Ad vector. To distinguish between the two mechanisms, large-scale preparations of AdRP1009 (15.1 kb), AdRP1005 (21.3 kb), and AdRP1001 (29.7 kb) were made, the resulting vectors were fractionated on a CsCl density gradient, and virion DNA was analyzed by restriction and Southern analyses. Fractions were collected through the viral bands, and each of these fractions was assayed for the presence of β-galactosidase- and luciferase-transducing particles (Fig. 4). Luciferase activity is expressed by the AdLC8cluc helper virus. AdRP1001 was partially separated from the helper virus due to the difference in density caused by the difference in its DNA content (29.7 kb) and that of the helper virus AdLC8cluc (35.4 kb) (Fig. 4A), as has been previously observed (25). Both AdRP1005 and AdRP1009 should band at a lower density than AdRP1001 due to their lower DNA content; however, this was not observed (Fig. 4B and C). AdRP1005 and AdRP1009 migrated to a CsCl density between that of AdRP1001 and AdLC8cluc, suggesting that the AdRP1005 and AdRP1009 virions contained DNA of between 29.6 and 35.4 kb, rather than 15 and 21.3 kb, respectively, as would be predicted based on the size of the original transfecting plasmid.

DNA was extracted and purified from virions from the peak fractions of BFU for each vector and analyzed by restriction digestion and Southern blot hybridization (Fig. 5B). Uncut vector DNA was also analyzed, and the results indicated that neither AdRP1009 (15.1-kb initial size) nor AdRP1005 (21.3-kb initial size) was of the predicted size based on the original transfecting plasmid, but rather, both of these vectors had an apparent size of greater than 23 kb (Fig. 5B, lanes 1 and 2). Upon overexposure of the autoradiograph, a minor band of approximately 15 kb was also observed in the lane containing

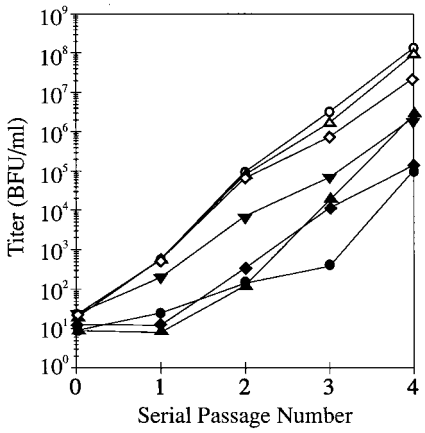

FIG. 3. Serial amplification of helper-dependent vectors. Aliquots of the crude vector lysates (500 μl per 60-mm-diameter dish) were used to infect 293Cre4 cells along with AdLC8cluc at an MOI of 1. The resulting crude vector lysates from the infected cells were assayed for the presence of BFU. The average of two amplification series is shown. Symbols: ○, AdRP1002 (33.5 kb); △, AdRP1001 (29.6 kb); ◇, AdRP1008 (27.7 kb); ●, AdRP1007 (25.7 kb); ▲, AdRP1006 (24.1 kb); ◆, AdRP1005 (21.4 kb); ▼, AdRP1009 (15.1 kb).

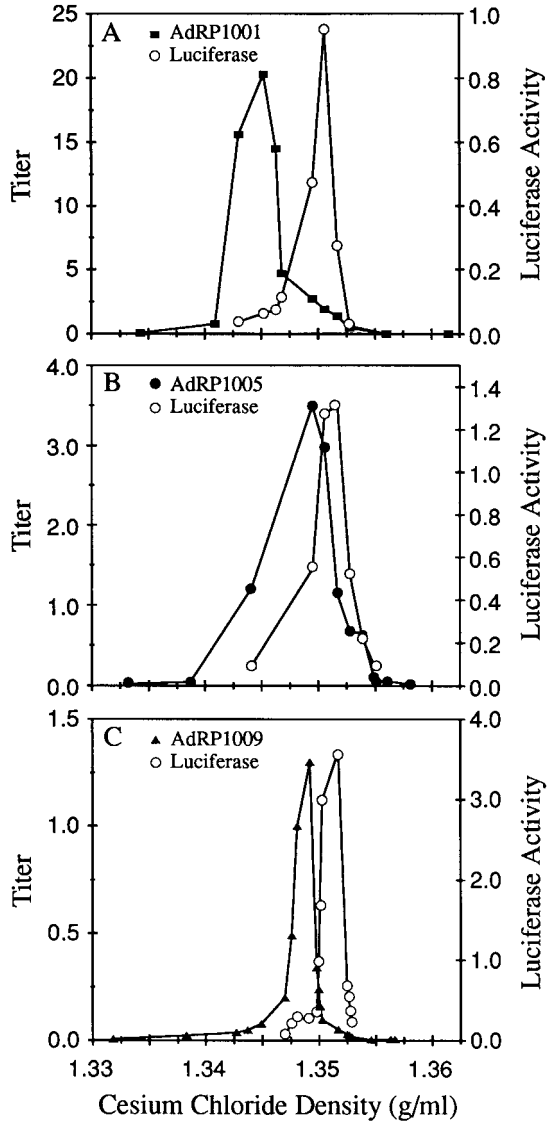

FIG. 4. CsCl purification of helper-dependent vectors AdRP1001, AdRP1005, and AdRP1009. Four 150-mm-diameter dishes of 293Cre4 cells were infected at an MOI of 1 with AdLC8cluc and the helper-dependent vector. After complete CPE, crude vector preparations were centrifuged to equilibrium on a CsCl buoyant-density gradient. Fractions were collected through the vector bands and assayed for vector titer (10^9 BFU/ml), and the ability to transduce luciferase (10^7 relative light units/ml). The density of the CsCl (g/ml) was determined using a refractometer.

uncut DNA from AdRP1009 and this may represent the packaging of two noncovalently joined monomer molecules of AdRP1009 or small amounts of contamination with virions containing only a single copy of the monomer AdRP1009 genome. Analysis of vector DNA restricted with *Hin*dIII showed that the restriction pattern obtained for AdRP1009 (original size of 15.1 kb, with predicted *Hin*dIII restriction fragments of 11.1, 2.9, and 1.0 kb) was consistent with a structure in which the virion DNA had formed covalently joined head-to-tail and tail-to-tail concatemers, creating additional diagnostic restriction fragments of 12.1 and 2.0 kb, respectively (Fig. 5B, lane 4, and 5A). The relative intensities of the 11.1- and 12.1-kb bands suggest that the two different dimer conformations were present in equal quantities (i.e., 3:1 molar ratio of a 11.1- to 12.1-kb fragment). Note that tail-to-tail dimers would not be packageable, and the 22-kb fragment diagnostic of such a structure was not detected. An additional band of 3.3 kb, of unknown origin, was also observed. Thus, starting with vector DNA which is approximately half the size required for efficient packaging in virions, successive passages of the virus resulted in selection for viral DNA that had formed concatemers. Viral DNA from AdRP1005, with predicted *Hin*dIII restriction fragments of 12.4, 5.1, 2.9, and 1.0 kb, generated a vector that had rearranged to a more complex structure(s), with an overall size of greater than 30 kb (Fig. 5B, lane 5). As expected, the restriction pattern for AdRP1001, with a genome that is larger than the lower cutoff for Ad packaging efficiency, was identical in structure to that predicted from the original transfecting plasmid (Fig. 5B, lane 6) and had therefore not undergone rearrangement. We conclude that Ad virions package viral DNAs of greater than 75% of the wild-type genome with equal efficiency; however, DNAs of smaller than 75% tend to undergo DNA rearrangement, resulting in a net increase in the size of the vector to greater than this lower limit, before efficient packaging.

Ads have a fairly well-defined upper packaging limit of approximately 105% of the wild-type genome size (4). We have now shown that Ads also possess a lower packaging limit corresponding to approximately 75% of the wild-type genome length. Although DNAs with sizes of less than this minimum are packaged, they apparently do so at a lower efficiency, resulting in a reduced virus recovery. When amplifying a vector of approximately 5.5 kb in a helper-dependent system, Fisher et al. (13) observed that the vector DNA had undergone DNA rearrangements and multimerization. Similar results were found by Haecker et al. (20). This is consistent with our results showing that vectors of less than ~27 kb are packaged inefficiently. Vector DNA which had undergone rearrangements, resulting in a net increase in size above this lower limit, would be strongly selected and would likely outgrow the smaller vector.

The range of DNA sizes encapsidated by Ads is consistent with results with other icosohedral virions, such as bacteriophage lambda which can package between 72 and 105% of the wild-type genome (28). Optimum DNA packaging lengths have also been observed for bacteriophage P1 (9) and Epstein-Barr virus (5). Taken together, these observations suggest that size limits within the 75 to 105% range may be a general characteristic of icosohedral viruses, including Ads.

Interestingly, we observed that a vector that was slightly less than half the size of the wild-type Ad genome was purified after amplification primarily as a covalent dimer. We predict that vector DNA of fractional size of the optimal packaging size (e.g., 1/3, 1/4, etc.) would therefore tend to form covalently joined multimeric species (e.g., trimeric, tetrameric, etc.). This might be advantagous for the development of vectors to be used in gene therapy since less virus would be required to attain the same level of expression of the foreign protein, due to the presence of multiple copies of the gene. Similarly, vectors could be constructed that contain two or more tandem copies of a foreign gene, and this arrangement might be stably maintained if the vector size was only slightly higher than the 27-kb lower packaging limit. For example, an Ad vector could be constructed with three copies of a 10-kb promoter:transgene cassette, for an overall size of ~30 kb. Unequal DNA recombination, resulting in an alteration of transgene copy number (e.g., one vector with four copies of the transgene and a second vector with only two copies), would produce vectors that either exceed the upper or are below the lower Ad DNA packaging limits. The clear demonstration of a lower packag-

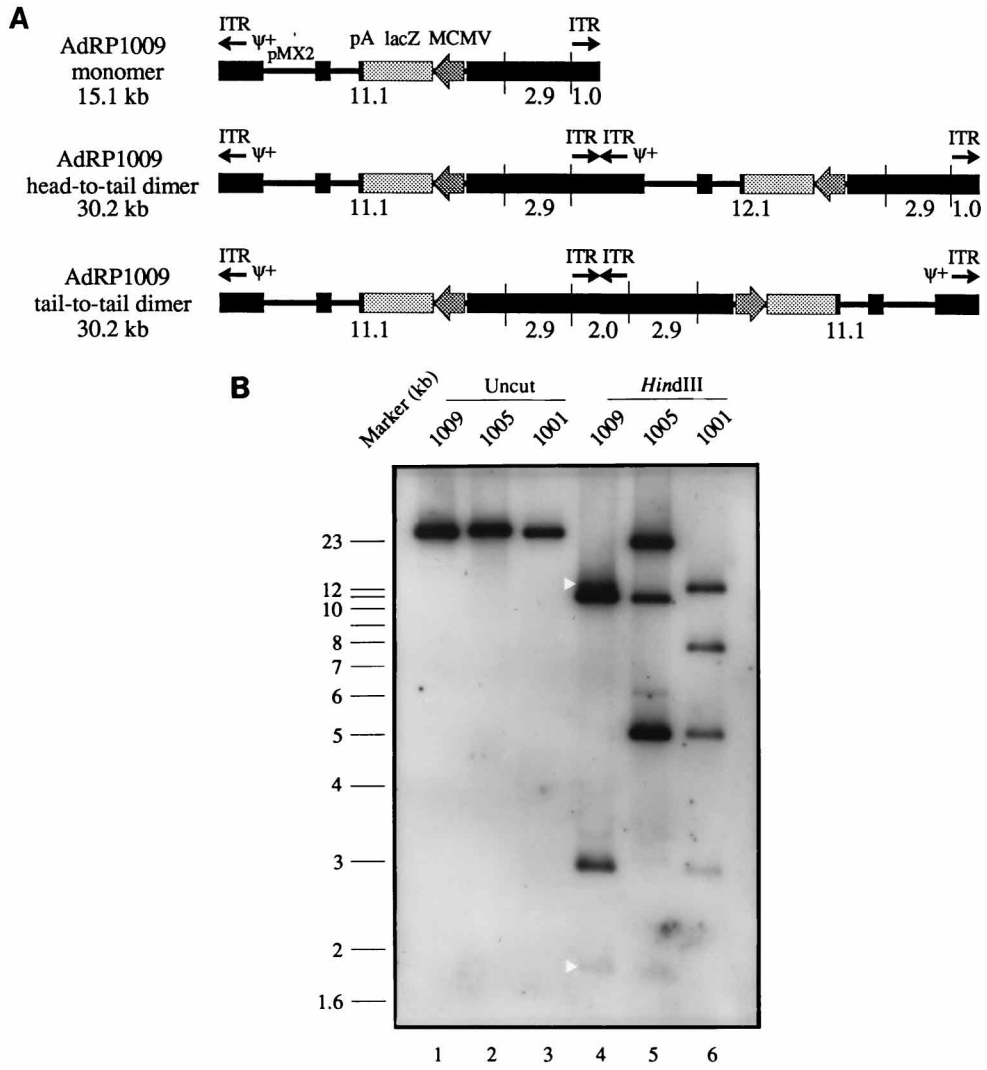

FIG. 5. (A) Predicted *Hin*dIII restriction maps of AdRP1009 monomer and head-to-tail or tail-to-tail covalent dimer. The sizes of the *Hin*dIII restriction fragments (in kilobases) are indicated below the schematic map. (B) DNA analysis of purified AdRP1009, AdRP1005, and AdRP1001. Aliquots from the peak of BFU activity corresponding to 7×10^6 BFU were digested overnight in pronase-sodium dodecyl sulfate buffer, extracted with phenol, and precipitated with ethanol. The resulting DNA was digested with *Hin*dIII, fractionated on a 0.8% agarose gel, transferred to a nylon membrane, hybridized to a digoxigenin-labelled pRP1001 DNA probe, and visualized by chemiluminescent reaction (DIG High Prime DNA labeling and detection kit; Boehringer Mannheim). The predicted *Hin*dIII restriction fragment sizes for the vectors are: AdRP1009 (11.1, 2.9, and 1.0 kb), AdRP1005 (12.4, 5.1, 2.9, and 1.0 kb), and AdRP1001 (12.1, 8.0, 5.1, 2.9, 1.0, and 0.6 kb). Restriction fragments smaller than 1.6 kb were visible upon overexposure of the autoradiograph (data not shown). The 12.1- and 2.0-kb bands from *Hin*dIII-digested AdRP1009 DNA, indicative of covalent head-to-tail and tail-to-tail dimer formation, are indicated with white arrowheads (lane 3).

ing limit for Ads will permit the design of vectors that are more stable and may lead to the development of vectors that express multiple copies of the foreign gene, requiring lower virus loads to attain the same level of expression as existing Ad vectors.

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REFERENCES

1. **Amalfitano, A., C. R. Begy, and J. S. Chamberlain.** 1996. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. Proc. Natl. Acad. Sci. USA **93:**3352–3356.
2. **Armentano, D., C. C. Sookdeo, K. M. Hehir, R. J. Gregory, J. A. St. George, G. A. Prince, S. C. Wadsworth, and A. E. Smith.** 1995. Characterization of an adenovirus gene transfer vector containing an E4 deletion. Hum. Gene Ther. **6:**1343–1353.
3. **Berkner, K. L.** 1992. Expression of heterologous sequences in adenoviral vectors. Curr. Top. Microbiol. Immunol. **158:**39–66.
4. **Bett, A. J., L. Prevek, and F. L. Graham.** 1993. Packaging capacity and stability of human adenovirus type 5 vectors. J. Virol. **67:**5911–5921.
5. **Bloss, T. A., and B. Sugden.** 1994. Optimal lengths of DNAs encapsidated by Epstein-Barr virus. J. Virol. **68:**8217–8222.
6. **Bramson, J. L., F. L. Graham, and J. Gauldie.** 1995. The use of adenoviral vectors for gene therapy and gene transfer *in vivo*. Curr. Opin. Biotechnol. **6:**590–595.
7. **Brody, S. L., and R. G. Crystal.** 1994. Adenovirus-mediated *in vivo* gene transfer. Ann. N. Y. Acad. Sci. **716:**90–101.
8. **Chen, L., M. Anton, and F. L. Graham.** Production and characterization of human 293 cell lines expressing the site specific recombinase Cre. Somatic Cell Mol. Genet., in press.
9. **Coren, J. S., J. C. Pierce, and N. Sternberg.** 1995. Headful packaging revisited: the packaging of more than one DNA molecule into a bacteriophage P1 head. J. Mol. Biol. **249:**176–184.
10. **Dong, J.-Y., D. Wang, F. W. Van Ginkel, D. W. Pascual, and R. A. Frizzell.**

1996. Systemic analysis of repeated gene delivery into animal lungs with a recombinant adenovirus vector. Hum. Gene Ther. **7:**319–331.
11. **Engelhardt, J. F., X. Ye, B. Doranz, and J. M. Wilson.** 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc. Natl. Acad. Sci. USA **91:**6196–6200.
12. **Engelhardt, J. F., R. H. Simon, Y. Yang, M. Zepeda, S. Weber-Pendelton, B. Doranz, M. Grossman, and J. M. Wilson.** 1993. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. Hum. Gene Ther. **4:**759–769.
13. **Fisher, K. J., H. Choi, J. Burda, S.-J. Chen, and J. M. Wilson.** 1996. Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. Virology **217:**11–22.
14. **Gilgenkrantz, H., D. Duboc, V. Juillard, D. Couton, A. Pavirani, J. G. Guillet, P. Briand, and A. Kahn.** 1995. Transient expression of genes transduced in vivo into heart using first-generation adenoviral vectors: role of the immune response. Hum. Gene Ther. **6:**1265–1274.
15. **Goldman, M. J., L. A. Litzky, J. F. Engelhardt, and J. M. Wilson.** 1995. Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2a-defective recombinant adenoviruses: a preclinical toxicology study. Hum. Gene Ther. **6:**839–851.
16. **Gorziglia, M. I., M. J. Kadan, S. Yei, J. Kim, G. M. Lee, R. Luthra, and B. C. Trapnell.** 1996. Elimination of both E1 and E2a from adenovirus vectors further improves prospects for in vivo human gene therapy. J. Virol. **70:**4173–4178.
17. **Grable, M., and P. Hearing.** 1992. *cis* and *trans* requirements for selective packaging of adenovirus type 5 DNA. J. Virol. **66:**723–731.

17a. **Graham, F. L.** 1984. Covalently closed circles of human adenovirus DNA are infectious. EMBO J. **3:**2917–2922.

18. **Graham, F. L., and L. Prevec.** 1992. Adenovirus-based expression vectors and recombinant vaccines, p. 363–390. *In* R. W. Ellis (ed.), Vaccines: new approaches to immunological problems. Butterworth-Heinemann, Boston, Mass.
19. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74.
20. **Haeker, S. E., H. H. Stedman, R. J. Balice-Gordon, D. B. J. Smith, J. P. Greelish, M. A. Mitchel, A. Wells, H. L. Sweeney, and J. M. Wilson.** 1996. In vivo expression of full length human dystrophin from adenoviral vectors deleted of all viral genes. Hum. Gene Ther. **7:**1907–1914.
21. **Horwitz, M. S.** 1990. Adenoviridae and their replication, p. 1679–1721. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York, N.Y.
22. **Kochanek, S., P. R. Clemens, K. Mitani, H.-H. Chen, S. Chan, and C. T. Caskey.** 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb independently expressing both full-length dystrophin and β-galactosidase. Proc. Natl. Acad. Sci. USA **93:**5731–5736.
23. **Krougliak, V., and F. L. Graham.** 1995. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. Hum. Gene Ther. **6:**1575–1586.
24. **Kumar-Singh, R., and J. S. Chamberlain.** 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. Hum. Mol. Genet. **5:**913–921.
25. **Mitani, K., F. L. Graham, C. T. Caskey, and S. Kochanek.** 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. Proc. Natl. Acad. Sci. USA **92:**3854–3858.
26. **Parks, R. J., L. Chen, M. Anton, U. Sankar, M. A. Rudnicki, and F. L. Graham.** 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc. Natl. Acad. Sci. USA **93:**13565–13570.

26a. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

27. **Schaak, J., X. Guo, W. Y.-W. Ho, M. Karlok, C. Chen, and D. Ornelles.** 1995. Adenovirus type 5 precursor terminal protein-expressing 293 and HeLa cell lines. J. Virol. **69:**4079–4085.
28. **Sternberg, N., D. Hamilton, L. Enquist, and R. Weisberg.** 1979. A simple technique for isolation of deletion mutants of phage lambda. Gene **8:**35–51.
29. **Yang, Y., F. A. Nunes, K. Berencsi, E. Gonczol, J. F. Engelhardt, and J. M. Wilson.** 1994. Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. Nat. Genet. **7:**362–369.
30. **Yang, Y., Q. Li, H. C. J. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.
31. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.
32. **Yeh, P., J.-F. Dedieu, C. Orsini, E. Vigne, P. Denefle, and M. Perricaudet.** 1996. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a 293-derived cell line expressing a minimal E4 functional unit. J. Virol. **70:**559–565.