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Genetics

Use of adeno-associated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into mammalian tissue culture cells

(provirus/adenovirus/gene transfer/inductible DNA rescue/recombinant parvovirus)

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ABSTRACT **Adeno-associated virus (AAV) is a human DNA virus that has a broad host range and can be grown both as an integrated provirus and as a lytic genome. These properties suggested that AAV may be useful as a mammalian transduction vector. To test this possibility, we have isolated a recombinant AAV viral stock in which the neomycin resistance gene was substituted for the AAV capsid genes. Using this recombinant stock, we have demonstrated that AAV can be used to transduce foreign DNA into human and murine tissue culture cells. In addition, we have demonstrated that, if the transductants are superinfected with a helper virus (adenovirus), the recombinant AAV genome is rescued from the proviral state and amplified to high copy number. These unique features of AAV vectors suggest that they may have a broad utility in the study of biological problems. Because AAV, itself, is nonpathogenic in both humans and animals, these vectors also may be useful for the purpose of gene therapy.**

Adeno-associated virus (AAV)-2 is a human parvovirus that can be propagated both as a lytic virus and as a provirus (1, 2). The viral genome consists of linear single-stranded DNA (3), 4675 bases long (4), flanked by inverted terminal repeats of 145 bases (5). For lytic growth AAV requires coinfection with a helper virus. Either adenovirus (6–8) or herpes simplex virus (9) can supply helper function and without helper, there is no evidence of AAV replication or transcription (10–12). When no helper virus is available, AAV can persist as an integrated provirus (2, 13–16). Integration apparently involves recombination between AAV termini and host sequences and most of the AAV sequences remain intact (15, 16) in the provirus. The ability of AAV to integrate into host DNA is apparently a strategy for insuring the survival of AAV sequences in the absence of the helper virus (16). When cells carrying an AAV provirus are subsequently superinfected with a helper, the integrated AAV genome is rescued and a productive lytic cycle occurs (2).

Much of the recent genetic work with AAV (17–19) has been facilitated by the discovery that AAV sequences that have been cloned into prokaryotic plasmids are infectious (17). When the wild-type (wt) AAV/pBR322 plasmid pSM620 is transfected into human cells in the presence of adenovirus, the AAV sequences are rescued from the plasmid and a normal AAV lytic cycle ensues (17). This means that it is possible to modify the AAV sequences in the recombinant plasmid and then to grow a viral stock of the mutant by transfecting the plasmid into human cells (18, 19). Using this approach, we have demonstrated the existence of at least three phenotypically distinct regions (Fig. 1) in AAV (19). The *rep* region codes for one or more proteins that are required for DNA replication and for rescue from the recombinant plasmid. The *cap* and *lip* regions (Fig. 1) appear to code for AAV capsid proteins and mutants within these regions are capable of DNA replication (19). In addition, studies of terminal mutants show that the AAV termini are required for DNA replication (18).

In this study, we isolate a recombinant AAV viral stock that carries a dominant selectable marker. Using this stock, we demonstrate that AAV can be used to introduce foreign DNA into mammalian cells. Furthermore, we show that when the transduced cells are superinfected with adenovirus, the integrated recombinant genome can be rescued and amplified. These experiments suggest that AAV may be useful as a generalized transduction vector with the unique property that the transduced sequences can be easily recovered from infected cells.

MATERIALS AND METHODS

Human Detroit 6 cells (D3405, hereafter called D6) and D5 cells (a D6-derived cell line that is latently infected with wt AAV and was previously called B7374IIIDV) were obtained from M. D. Hoggan (National Institutes of Health). Human KB cells were a gift from H. Ginsberg (Columbia University); 293-31 cells, a human adenovirus-transformed cell line, was a gift from T. Shenk (Stony Brook); and mouse L thymidine kinase negative (tk^-) cells were obtained from S. Silverstein (Columbia University). All cells were grown as described (19). Enzymes used in this study were purchased from New England Biolabs or Bethesda Research Laboratories and used as suggested by the supplier. *Bgl* II linkers were purchased from New England Biolabs; *Bam*HI linkers were purchased from Collaborative Research. DNA transfections, virus infections, and wt titer determinations were as described (21, 22). DNA extractions and Southern hybridization procedures have also been described (21, 23, 24).

Preparation of Recombinant Virus Stock. Five micrograms of *dl*52-91/neo or *dl*52-91/dhfr plasmid DNA and 0.5 μg of *ins*96/λ-M plasmid DNA were cotransfected into adenovirus 2 (Ad2)-infected [multiplicity of infection (moi) = 5] KB or 293-31 cells by using DEAE-dextran (25) as described (21). The transfected cells were treated with 0.1 mM chloroquin diphosphate (26) for 4 hr (KB cells) or 20 min (293-31 cells). Two days after transfection, the cells were frozen and thawed twice and passed through a 0.45-μm filter to remove cellular debris. The contaminating Ad2 helper virus was inactivated by heating the virus stock at 56°C for 2 hr.

Construction of Recombinant Plasmids. The construction of *dl*52-91/dhfr and *dl*52-91/neo is illustrated in Fig. 2. The plasmids pBR-neo (27) and pSV-dhfr (20) were obtained from P. Berg (Stanford University). *ins*96/λ-M was derived

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Abbreviations: AAV, adeno-associated virus; SV40, simian virus 40; Ad2, adenovirus type 2; m.u., map unit(s); kb, kilobase(s); neo, neomycin; dhfr, dihydrofolate reductase; wt, wild-type; ^{r}, resistance; moi, multiplicity of infection.

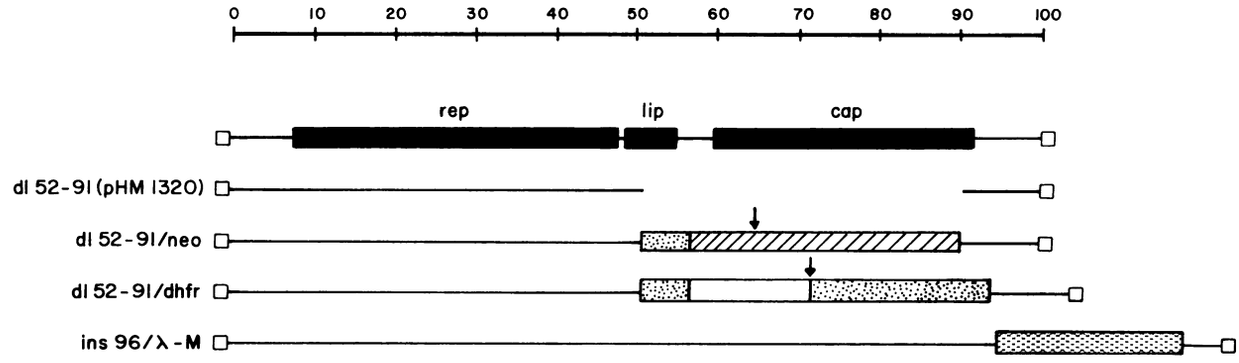

FIG. 1. Physical organization of AAV recombinant genomes. Solid boxes indicate the position of the coding sequences for the *rep*, *lip*, and *cap* functions of AAV. These were determined from the position of AAV mutants (19). The interruption in the *dl*52-91 line indicates the position of the deletion in this mutant. *dl*52-91 also has a single *Bgl* II site that has been inserted in place of the deleted sequences. *dl*52-91/neo contains the simian virus 40 (SV40) origin and promoter sequences [SV40 map units (m.u.) 71–65, stippled box] and the neomycin (*neo*) resistance gene sequences (striped box) inserted in *dl*52-91. In the process of constructing *dl*52-91/neo, the *Bgl* II site in the vector was lost (see Fig. 2). The arrow indicates the single *Bgl* II site [within the neo-resistance (neo^r) sequence] that is present in the construct. *dl*52-91/dhfr (dhfr = dehydrofolate reductase) was constructed by inserting the SV40/*dhfr* sequences from the plasmid pSV2-dhfr (20) into *dl*52-91 (see Fig. 2). Stippled boxes represent SV40 sequences, the open box represents the mouse *dhfr* coding sequence, and the arrow indicates a unique *Bgl* II site. *ins*96/λ-M was constructed by inserting a 1.1-kilobase (kb) bacteriophage λ fragment (open box) into the single *Bgl* II site of *ins*96.

from the plasmid pSM620*Xba*I, kindly supplied to us by R. J. Samulski (Stony Brook; personal communication). The pSM620*Xba*I plasmid is a derivative of pSM620 (17), which contains an *Xba* I linker inserted at AAV m.u. 96. The *Xba* I site of pSM620*Xba*I was converted to a *Bgl* II site by inserting a *Bgl* II linker and the resulting plasmid was called *ins*96(pHM2904). Finally, a 1.1-kb *Sau*3A bacteriophage λ fragment was inserted into *ins*96 to produce *ins*96/λ-M.

Transduction of Mammalian Cells. D6, KB, or Ltk^- cells were plated at 10^2 to 10^5 cells per dish. Cells were infected with *dl*52-91/neo virus 12–24 hr after plating and selected for drug resistance at 12–24 hr or 7 days after infection. D6 and KB cells were selected with 1 mg of Geneticin [G418 sulfate (28) (GIBCO)] per ml; L cells were selected at 0.4 mg/ml. These antibiotic concentrations were chosen because they killed all of the uninfected cells in 6–10 days. Cells were fed every 3–4 days. At 8–14 days after selection, cells were fixed with ethanol, stained with Giemsa, and counted.

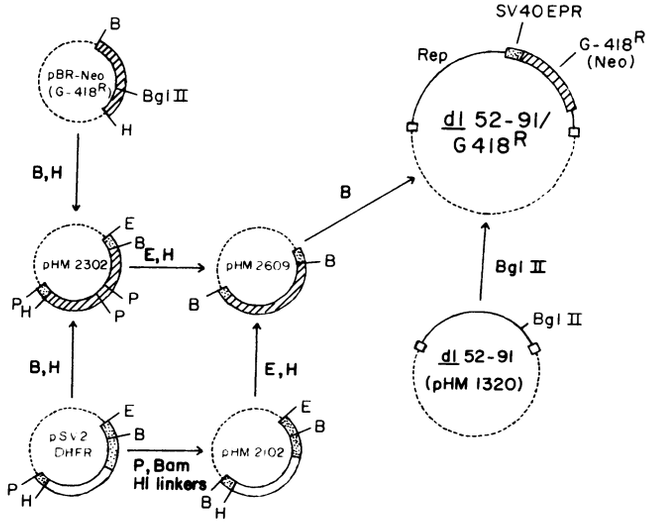

FIG. 2. Construction of AAV recombinants. Intermediate steps in the construction of *dl*52-91/neo (or $G418^r$). Arrows indicate the derivation of each plasmid as well as the restriction enzymes that were used at each step. For example, pHM2302 was the product of a ligase reaction that contained the *Bam*HI (B) and *Hin*dIII (H) double-digest fragments of pBR-neo (27) and pSV2-dhfr (20). Other enzyme designations are *Eco*RI (E) and *Pvu* II (P). As in Fig. 1, stippled boxes are SV40 sequences, open boxes are mouse *dhfr* sequences, and striped boxes are *neo* sequences. Solid lines and small open boxes represent AAV coding and terminal sequences, respectively; dotted lines represent pBR322 sequences. *dl*52-91/dhfr was constructed by inserting the appropriate *Bam*HI fragment from pHM2102 into *dl*52-91 (not shown).

RESULTS

Construction of an AAV/Neo Recombinant. To test the possibility of using AAV as a transducing virus, it was necessary to insert a selectable marker into the AAV genome. For this purpose, we chose the *neo* resistance gene under the control of an SV40 promoter (28). Briefly, the SV40 sequences (SV40 m.u. 71–65) were excised from the plasmid pSV2-dhfr (20) and the *neo* resistance gene was obtained from the pBR-neo (27) plasmid (Fig. 2). These two regions were inserted into the AAV deletion mutant *dl*52-91 in the orientation shown in Fig. 1. As the name implies, *dl*52-91 is missing the AAV sequences between m.u. 52 and 91 (Fig. 1). It is, therefore, capable of DNA replication but, because it does not contain the AAV capsid genes, it is incapable of producing infectious virus (19). Based on the orientation of the inserted sequences, we expected the *neo* gene to be expressed as the result of transcription that was initiated within the SV40 early promoter sequences and terminated at the AAV polyadenylylation signal (m.u. 95). The size of the *dl*52-91/neo recombinant was 4.7 kb, which is essentially the same as that of wt DNA.

A second recombinant in which the mouse *dhfr* gene was inserted into *dl*52-91 was also constructed (Fig. 1). This recombinant, *dl*52-91/dhfr, is 6% larger than the wt AAV genome.

To determine if the recombinant plasmids (neo and dhfr) could replicate, the two plasmid DNAs were transfected into Ad2-infected human cells. As expected, both the neo and the dhfr recombinants were found to replicate to approximately the same extent as the wt AAV (Fig. 3). Digestion with *Bgl* II (which does not cut wt AAV) of DNA rescued from cells after transfection with *dl*52-91/neo confirmed the structure of the neo recombinant (Fig. 3).

Preparation of Neo-Transducing Virus Stock. To grow a *dl*52-91/neo viral stock it was necessary to supply the missing AAV capsid gene products in *trans*. Initial attempts to isolate a stock by complementation with the wt plasmid pSM620 were unsuccessful. For reasons that were not clear, there was a strong bias toward packaging the wt genome.

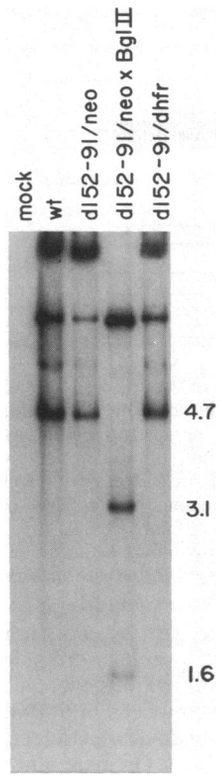

FIG. 3. Comparison of wt and recombinant DNA replication. One microgram of the indicated form I supercoiled plasmid DNA was transfected into KB cells. The cells were subsequently infected with Ad2 helper virus at a moi of 5. At 24 hr, cells were lysed and treated with Pronase and low molecular weight DNA was isolated by Hirt (23) extraction. One-tenth of each DNA extract was then fractionated on a 1.4% agarose gel, transferred to nitrocellulose by the method of Southern (24), and hybridized to nick-translated *dl*52-91/neo DNA. Where indicated, the extract was digested with *Bgl* II. The large *Bgl* II fragment (3.1 kb) of *dl*52-91/neo has approximately the same amount of sequence homology to the probe as the full-size band of wt AAV DNA (4.7 kb). Bands that are larger than 4.7 kb are normal concatemeric replication intermediates that accumulate during AAV DNA replication or input plasmid species. The identity of the *dl*52-91/neo DNA was confirmed by digestion with restriction enzymes other than *Bgl* II (not shown).

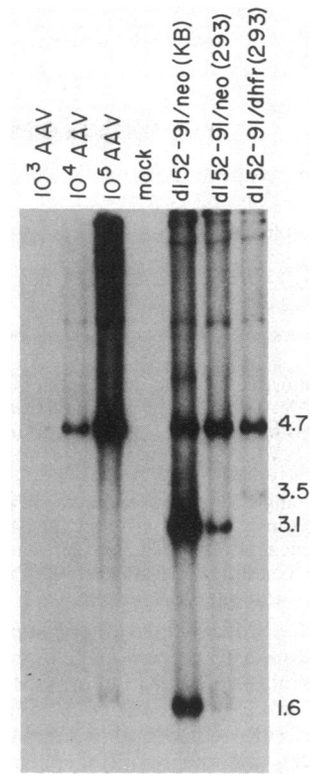

FIG. 4. Transducing virus titer determination. Ad2-infected D6 cells (10^7 cells per plate) were either infected with known quantities of wt AAV virus or 100 μl of one of three recombinant virus stocks. At 48 hr, DNA was harvested, digested with *Bgl* II, and analyzed as in Fig. 3. Each lane represents 10% of the DNA recovered from one 10-cm dish. The lane marked mock contains DNA from uninfected cells. *dl*52-91/neo (KB) and *dl*52-91/neo (293) were virus stocks grown in KB and 293 cells, respectively. The lanes marked AAV contain DNA from cells that had been infected with the indicated number of infectious units, as determined by immunofluorescence. The probe was nick-translated *dl*52-91/neo plasmid DNA.

To eliminate the packaging bias, an AAV plasmid was constructed that was too large to be packaged. This was done by inserting a 1.1-kb fragment of bacteriophage λ DNA into a nonessential region of AAV (R. J. Samulski and T. Shenk, personal communication) at m.u. 96. The resulting plasmid *ins*96/λ-M (Fig. 1) contained all of the AAV coding regions intact. When the *dl*52-91/neo and *ins*96/λ-M plasmid DNAs were transfected into human cells at a 10:1 ratio, viral stocks were obtained that were enriched for the *dl*52-91/neo-transducing virus (Fig. 4). Although the neo viral stocks contained none of the complementing *ins*96/λ-M genome, a variable number of the virions apparently contained wt AAV DNA. The appearance of the wt genome was presumably the result of recombination between *ins*96/λ-M and *dl*52-91/neo. Recombination between two complementing AAV mutants following DNA transfection had been observed previously and was expected (19).

The *dl*52-91/neo viral stocks were titered by comparing the yield of replicating AAV DNA obtained following infection of human cells against wt infections using a titered AAV viral stock (Fig. 4). The wt stock, in turn, was titered by immunofluorescent focus formation using anti-AAV capsid antibody (22). This rather indirect approach to titering recombinant stocks was necessary because AAV does not plaque by itself and also because only AAV anti-capsid antibodies are currently available. As shown in Fig. 4, the titer of the *dl*52-91/neo stocks varied depending on the host cell that was used to grow them and on the input ratio of neo to complementing plasmid DNA (not shown). One of our stocks, *dl*52-91/neo (KB), was estimated to have $\approx 10^6$ infectious units/ml of *dl*52-91/neo virions and 2×10^5 infectious units/ml of wt AAV (Fig. 4). This stock was chosen for further experiments. As expected, we had less success in obtaining a high-titer *dl*52-91/dhfr stock, presumably because this DNA was larger than wt size.

Transduction of Tissue Culture Cells with *dl*52-91/Neo Virus. Human D6 cells were infected at various moi (0.1–1000) and then incubated in the presence of the antibiotic G418. When the G418 drug selection was applied at 12–24 hr after AAV/neo infection, the frequency of transduction in D6 cells appeared to be ≈1% (Table 1). Furthermore, the transduction frequency was relatively constant with respect to the moi. A 10^4-fold increase in moi produced an 8-fold increase in transduction. Two other cell lines, human KB cells and mouse Ltk^- cells, were also capable of being transduced, although at a somewhat lower frequency.

To see if the frequency of transduction might be a function of the time at which selection was applied, human D6 cells were allowed to incubate for 7 days after infection before G418 was added to the media. When this was done (Table 1), the frequency of transduction was 10%.

Table 1. Transduction of tissue culture cells with *dl*52-91/neo virus

Cell line	G418^r-transducing virus, moi	Selection time, days	Transduction efficiency, %*
D6	0.1	1	0.4 (0.04)
D6	1	1	0.6 (0.4)
D6	100	1	1.3 (1.3)
D6	1000	1	3 (3)
D6	1000	7	10 (10)
KB	1	1	0.1 (0.07)
Ltk$^-$	0.4	1	0.05 (0.02)

The indicated cells were seeded at 10^2, 10^3, or 10^5 cells per dish and infected at the indicated moi. G418 selection was applied at the indicated time after infection (1 mg/ml for human cells or 0.4 mg/ml for murine cells). Colonies were stained with Giemsa and counted at days 8–14.

*Transduction efficiency equals the number of G418^r colonies divided by the number of infected cells. At low moi, the number of infected cells was calculated by using the Poisson distribution. Numbers in parentheses indicate the number of transductants divided by the number of cells seeded on the plate regardless of the moi.

Rescue of *dl*52-91/Neo from Transduced Cells. Human D6 cells (10^5) were infected with *dl*52-91/neo virus at a moi of 10, allowed to grow to confluence in the presence of G418, and then passaged twice in the presence of the drug (≈15–20 generations). The resulting cells probably represented a mixture of 10^3 individual transductants. This heterogeneous cell line was then expanded further and used to determine the state of the AAV/neo DNA within the transduced cells.

Examination of the low molecular weight DNA (23) indicated that there were no free (low molecular weight) AAV or *neo* sequences in the G418^r cells (Fig. 5). This was similar to what was seen with D5 cells, which are a D6-derived cell line known to be latently infected with wt AAV (Fig. 5; ref. 15). We take this to mean that the *dl*52-91/neo virus had induced transformation in human cells by integration of the recombinant genome into host chromosomes. Because the neo^r cell line described here was heterogeneous, it was not possible to directly demonstrate the presence of AAV recombinant sequences within genomic DNA. However, we have also isolated eight independent neo-transduced clones and in all of these, the restriction pattern of the AAV/*neo* sequences [as determined by Southern hybridization (24)] was consistent with the integration of one or more copies of the recombinant genome into host DNA (data not shown).

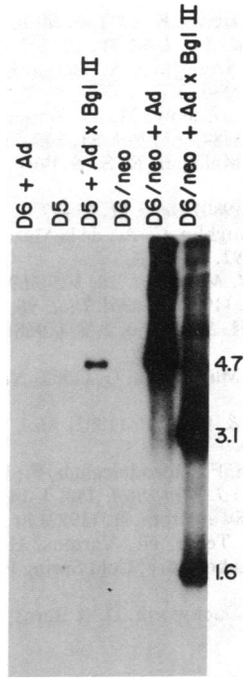

FIG. 5. Rescue of AAV and *neo* sequences from transformed cells. Parental D6 cells, D5 cells, or D6 cells transduced with *dl*52-91/neo virus (D6/neo) were examined for the presence of low molecular weight AAV or *neo* sequences as described in the legend to Fig. 3. Where indicated, the cells were infected with Ad2 48 hr before DNA isolation. Also, where indicated, the extracted DNA was digested with *Bgl* II prior to electrophoresis.

When the neo-transduced cells were superinfected with Ad2 (moi = 5), the *dl*52-91/*neo* sequences were rescued and amplified (Fig. 5). Digestion of the rescued DNA with *Bgl* II indicated that the bulk of the rescued sequences had the restriction pattern expected of the *dl*52-91/neo recombinant DNA. A small amount of wt AAV DNA (resistant to *Bgl* II) was also seen (Fig. 5) and was, presumably, due to the fact that wt AAV had been present in the *dl*52-91/neo virus stock. Similar results were seen when the D5 cell line was superinfected with Ad2, except that, as expected, the rescued wt AAV sequences were insensitive to *Bgl* II digestion. Essentially, then, it appeared that infection of human cells with *dl*52-91/neo virus could produce a latent infection in the same manner as wt AAV virus. Similarly, the latent neo genome behaved like integrated wt AAV DNA in that it could be rescued by superinfection of the carrier cells with adenovirus.

DISCUSSION

We have demonstrated that AAV can be used as a transducing virus for introducing foreign DNA into human and mouse tissue culture cells. Presumably, the transductants that were isolated were due to integration of the recombinant genome into host chromosomes. Preliminary experiments indicate that this is the case. Previous studies of the physical structure of wt integrated genomes by Cheung *et al.* (15) and Berns *et al.* (16) suggested that AAV integrates at random positions in the host chromosome but at a unique position with respect to its own DNA—i.e., within its terminal repeat sequence. This is quite similar to the behavior of retroviral proviral DNA (29) but it is not clear yet whether the mechanism of integration is the same. Studies of AAV integration have been hampered by the fact that wt AAV does not code for a dominant selectable marker (e.g., an *onc* gene). For this reason, the recombinant AAV virus described here should be useful. In our preliminary experiments, we observed transduction frequencies of 0.4–10% in human cells and a somewhat lower frequency in murine cells. It was not clear whether these values reflected the ability of the AAV recombinant to integrate or the ability of the *neo* gene to be expressed in these different genetic environments. The fact that the transduction frequencies were relatively constant with respect to moi suggests that a single recombinant genome may be sufficient for transduction. This has been confirmed by genomic Southern blots on neo^r clones isolated after low-multiplicity infections (unpublished).

Although the primary focus of this report was to demonstrate that AAV can be used to transduce foreign genes into mammalian cells, AAV vectors have a number of interesting features that should be pointed out.

***(i)* Expression of Integrated AAV Genomes.** Normally, integrated AAV genomes are silent. In the absence of a helper virus (i.e., adenovirus) there is no evidence of AAV-specific transcription in either latently infected cells or in noncarrier cells that have been infected with AAV (10, 30). It is not clear whether this is due to repression of AAV transcription or the need for positive activation by a helper virus-coded

gene product. Whatever the reason, however, it did appear from our transduction experiments that when a foreign gene is inserted into AAV it can be expressed, provided that an appropriate promoter is present. Although an SV40 promoter was used in these experiments, there is no reason to believe that other promoters could not have worked as well. This suggests that AAV may be useful for gene replacement experiments in which it is desirable to have the foreign gene under the control of its own enhancer and promoter elements.

***(ii)* Rescue of AAV Genomes.** A unique feature of integrated AAV genomes is that they can be rescued when the host cell is infected with adenovirus. We have shown that this is true, as well, of recombinant AAV DNA (Fig. 5). This illustrates an interesting aspect of AAV vectors—namely, that they can be used both for inserting and recovering foreign DNA from mammalian cells. Our attempts to package the *dl*52-91/dhfr genome (as well as other recombinant DNAs; unpublished) suggest that the packaging limit of AAV virions is ≈5 kb. Genetic analysis of AAV mutants (18, 19) indicates that the only *cis*-active sequences required for AAV rescue and replication are the terminal repeats (145 base pairs), and, therefore, most of the AAV genome is available for the substitution of foreign DNA. Thus, AAV should be appropriate for experiments involving the cloning and expression of mammalian cDNA fragments as well as short genomic DNA fragments.

***(iii)* Studies of Larger DNA Fragments.** Our experiments have shown also that recombinant AAV DNA can be rescued from prokaryotic plasmids after DNA transfection (Fig. 3). Under these conditions, there is no obvious limit on the size of the foreign DNA insert. This means that in the presence of adenovirus, the infectious AAV plasmids can be used as transient expression vectors. Furthermore, rescue of AAV recombinants, presumably, would occur even if the plasmid DNA had integrated into a mammalian chromosome. This suggests that AAV vectors could be used in conjunction with other DNA transfer techniques (i.e., microinjection, protoplast fusion, calcium phosphate transfection) to rescue foreign DNA from mammalian cells.

***(iv)* Host Range.** Although AAV is believed to be a human virus, its host range for lytic growth is unusually broad. Virtually every mammalian cell line that has been tried can be productively infected with AAV provided an appropriate helper virus is used (1). The fact that we have successfully used AAV for the transduction of murine cells suggests that the host range for AAV integration is equally broad.

***(v)* Gene Therapy.** AAV vectors may be particularly useful for some types of gene therapy protocols. In this respect it is worth noting that (*a*) no disease has been associated with AAV in either human or animal populations (1, 30), (*b*) integrated AAV genomes are essentially stable in tissue culture for >100 passages (15), and (*c*) there is no evidence that integration of AAV alters the expression of host genes or promotes their rearrangement.

In summary, AAV appears to be an unusually flexible vector with several unique features. It should be useful, therefore, for the study of a variety of biological problems.

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