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Adeno-Associated Virus Vector for High-Frequency Integration, Expression, and Rescue of Genes in Mammalian Cells

JON-DURI TRATSCHIN,† IRVING L. MILLER, MERVYN G. SMITH,‡ AND BARRIE J. CARTER*

Laboratory of Molecular and Cellular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, Maryland, 20205

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We describe the construction of an adeno-associated virus (AAV) vector in which the coding sequence of the procaryotic gene *neo* is expressed under the control of the major AAV promoter p_{40}. This AAV-*neo* vector allowed stable expression of *neo* as a dominant selective marker in mammalian cells by selection of cells which were resistant to the antibiotic geneticin (G418). When the vector was introduced into human (293 or HeLa) cell lines by a DNA transfection procedure, stable geneticin-resistant colonies were obtained. When the vector was first packaged into AAV particles and then introduced into cells via particle infection, geneticin-resistant cells were obtained at higher frequencies than those obtained by DNA transfection. In geneticin-resistant cells the AAV-*neo* vector was integrated at low copy number and could be rescued by subsequent infection with wild-type AAV and the helper adenovirus or, in some cases, by infection with adenovirus alone. The rescued AAV-*neo* vector could then be recovered as amplified unintegrated DNA from a Hirt lysate. These results demonstrate that AAV can be used as a transducing viral vector for stable integration and expression of a foreign gene in mammalian cells. The high frequency of integration and the ability to rescue the integrated vector suggest that this vector system may be useful for selecting genes from cDNA libraries. This vector may also be useful for introduction of genes into cells which are refractory to transfection in procedures such as those involving the use of $CaPO_4$ or DEAE-dextran.

Studies of eucaryotic gene regulation have been greatly facilitated by the development of eucaryotic viral expression vectors. Vectors based on RNA retroviruses or on DNA viruses including papovaviruses, adenoviruses, herpesviruses, or poxviruses have been described (8, 13, 29, 34, 35).

The human parvovirus adeno-associated virus (AAV) grows in mammalian cells in the presence of helper functions provided by adenovirus or herpesvirus (reviewed in reference 5). Furthermore, AAV is limited for replication only by the host range of the helper and will grow in a wide variety of human, simian, and rodent cells if the appropriate helper virus is provided. In the absence of helper functions the AAV genome frequently integrates into the host cell chromosome with no apparent effect on cell growth or morphology (9, 12, 17, 22). Subsequent addition of helper virus allows excision of these integrated AAV genomes and replication to produce infectious AAV particles (22). Thus, AAV is a potential vector for introduction of genes into cells in either a chromosomal or an episomal (unintegrated) state.

When recombinant plasmids containing an AAV2 genome are transfected into mammalian cells in the presence of helper adenovirus, the AAV genome is rescued free of any plasmid DNA sequence and replicated to produce infectious AAV particles (26, 38, 39, 41). Also, molecular clones of AAV2 containing a foreign gene under the control of either of the AAV promoters p_{19} or p_{40} could be used as transient expression vectors when transfected into mammalian cells even in the absence of any helper virus (47). In particular, the AAV promoter p_{40} was a strong constitutive promoter in several cell lines.

We now show that AAV vectors using an AAV transcription promoter can be used for high-frequency integration, expression, and rescue of genes in mammalian cells. We used the procaryotic gene *neo*, which codes for a neomycin phosphotransferase II and mediates resistance to aminoglycoside antibiotics such as neomycin and kanamycin (2, 24). This gene can be used as a dominant selective marker in mammalian cells (10, 43) to provide resistance (*gen*^{r}) to the antibiotic geneticin (G418). When AAV vector DNA containing the *neo*-coding sequence under the control of the AAV promoter p_{40} was transfected into human cell lines, *gen*^{r} cells were obtained at a frequency of about 10^{-4}, but if the vector was first packaged into AAV-transducing particles the frequency of *gen*^{r} cells was increased by at least 2 orders of magnitude. The vector was integrated at low copy number into the cell genome in most cases and could be rescued by subsequent infection with helper viruses. Thus, the AAV promoter p_{40} is a very efficient promoter even when integrated into the cell chromosome. Hermonat and Muzyczka (18) also demonstrated that AAV can be used as an efficient transducing vector for expression of *gen*^{r}, but in their vector the *neo* sequence was expressed under the control of a simian virus 40 (SV40) early gene transcription promoter. The AAV vector provides a useful system for integration of genes into cell chromosomes at low copy number.

MATERIALS AND METHODS

Growth and purification of plasmids. The procedures for construction, growth, and purification of recombinant plasmids have been described previously (46, 47). All plasmids were grown and maintained in *Escherichia coli* HB101 in ampicillin-containing medium. To ensure high-quality DNA, all preparations were banded twice in

* Corresponding author.
† Present address: Institute for Hygiene and Medical Microbiology, University of Berne, Berne, Switzerland.
‡ Present address: Department of Biochemistry, University of Otago, Dunedin, New Zealand.

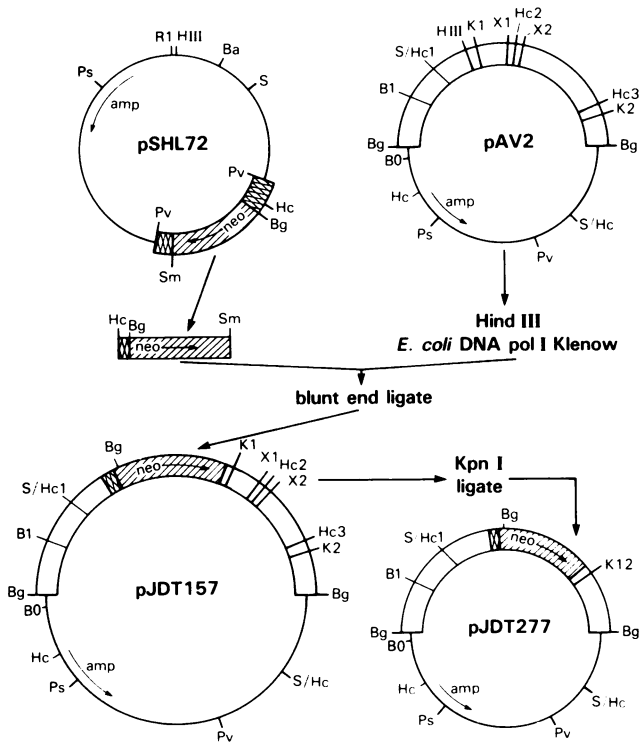

FIG. 1. Construction of AAV vectors containing the *neo* gene sequence. The circles indicate recombinant plasmids. The derivation of DNA sequences is designated as follows: single line, pBR322 DNA; double line, AAV2 DNA; diagonally shaded line, *neo* gene-coding sequence from bacterial transposon Tn*5*; cross-hatched, portions of the HSV TK gene. pSHL72 contains a *Bgl*II-*Sma*I fragment from Tn*5* containing the neomycin phosphotransferase II gene flanked by sequences from the HSV TK gene inserted at the*Pvu*II site of pBR322. The direction of transcription of the *neo* gene is indicated by the arrow. The HSV DNA sequences Pv-Hc and Sm-Pv contain the HSV TK gene promoter and polyadenylation sites, respectively. pAV2 contains the entire AAV2 genome inserted via *Bgl*II linkers in a plasmid derived from pBR322 (26). Relevant restriction endonuclease sites are shown and designated as follows: Ps, *Pst*I; RI, *Eco*RI; HIII, *Hin*dIII; B, *Bam*HI; S, *Sal*; Pv, *Pvu*II; Hc, *Hin*cII; Bg, *Bgl*II; Sm, *Sma*I; X, *Xho*I; K, *Kpn*I. For AAV, the B, X, K, and Hc sites are distinguished by an additional numeral, e.g., X1, X2. For Hc and B sites the plasmid site is enumerated as B1 or Hc.

CsCl-ethidium bromide gradients. All manipulations with recombinant DNA were performed in accordance with the National Institutes of Health Guidelines.

Construction of individual plasmids. The structure of pAV2 and the construction of certain plasmids are detailed in Fig. 1, and all of the individual plasmids used in this work are summarized in Table 1 and Fig. 2.

pSHL72 (49) was constructed by S. Larsen and was obtained from N. Jones, Purdue University, West Lafayette, Ind. This plasmid is a pBR322 derivative and contains the procaryotic coding sequence for the gene *neo* inserted within the transcription unit of the herpes simplex virus type I (HSV) gene for thymidine kinase (TK) such that the *neo* gene is expressed in eucaryotic cells under the control of the HSV TK promoter and polyadenylation signals (Fig. 1).

pAV2 (Fig. 1 and 2) comprises the AAV2 genome inserted via *Bgl*II linkers into a pBR322-derived plasmid, pA11P.Xba, at the *Bgl*II site of the polylinker sequence (28). pJDT95 was derived from pAV2 (45). pJDT243 was derived from pAV2 by deletion of the region between the *Bam*HI sites B0 and B1 (Fig. 2).

To construct the AAV-*neo* vectors (Fig. 1) a 970-base-pair *Hin*cII-*Sma*I fragment was excised from pSHL72 and inserted by blunt-end ligation into pAV2 DNA that had been cleaved with *Hin*dIII and incubated with *E. coli* DNA polymerase I Klenow fragment to fill in cohesive ends. The ligation mix was used to transfect *E. coli* HB101 and obtain the plasmid pJDT157. A second plasmid, pJDT114, which contained the *neo* gene inserted in the opposite orientation was also obtained. pJDT277 was derived from pJDT157 by deletion of the *Kpn*I fragment between the sites K1 and K2 (Fig. 1). pJDT174 was constructed similarly to pJDT157, but the *Hin*cII-*Sma*I fragment from pSHL72 was inserted by blunt-end ligation into pJDT95 (Fig. 2) which had been cleaved with *Xho*I. This resulted in insertion of the *neo* sequence downstream of the AAV intron. Similarly, pJDT177 has the *neo* sequence inverted with respect to pJDT174.

Cells and viruses. AAV2 and adenovirus type 2 were grown as described previously (6). Human 293-31 (adenovirus type 5-transformed human embryonic kidney) cells (15) and HeLa cells were grown at 37°C in monolayer culture in plastic dishes in Eagle minimal essential medium supplemented with antibiotics and 10% fetal calf serum. KB cells were grown in spinner culture (6). Cells were infected with adenovirus, AAV, or AAV-transducing particles by standard procedures (6).

Transfection of cells. 293 cells or HeLa cells grown in monolayers were transfected with plasmid DNA by the $CaPO_4$ procedure (50) exactly as described previously (46). When adenovirus was used, the cells were infected 1 h before transfection and the virus inoculum (20 PFU per cell) was not removed before addition of the DNA-$CaPO_4$ precipitate. The cell growth medium was replaced with fresh medium 4 h after transfection.

Packaging of AAV vectors in AAV-transducing particles. AAV-transducing particles containing the vector JDT277

TABLE 1. Plasmids used in this work[a]

Plasmid[b]	Genotype[b]	Relevant properties	Reference
pSHL72		*neo* behind HSV TK promoter	49
pAV2		AAV2 genome in pA11P.Xba	26
pJDT95	pAV*d*lHc23	Deletion of capsid antigen from pAV2	46
pJDT157	pAVHneo	*neo* in AAV, intron downstream	This work
pJDT114	pAVHineo	*neo* inverted relative to pJDT157	This work
pJDT277	pAVHneo*d*lK12	*neo* in AAV, intron deleted	This work
pJDT174	pAVXneo*d*lHc23	*neo* in AAV, intron upstream	This work
pJDT177	pAVXineo*d*lHc23	*neo* inverted relative to pJDT174	This work
pJDT243	pAV*d*lB0B1	AAV *rep*$^-$ mutant	This work

[a] The construction and detailed properties of the plasmids are described further in the text.

[b] The plasmids are generally referred to in the text by the plasmid name. For AAV plasmids we show also the genotype name, since this system of nomenclature was used in a previous publication (46). Note also that the prefix "P" refers only to the recombinant plasmid. The AAV genome contained within or excised from the plasmid is referred to by the plasmid name or the genotype name but without the prefix.

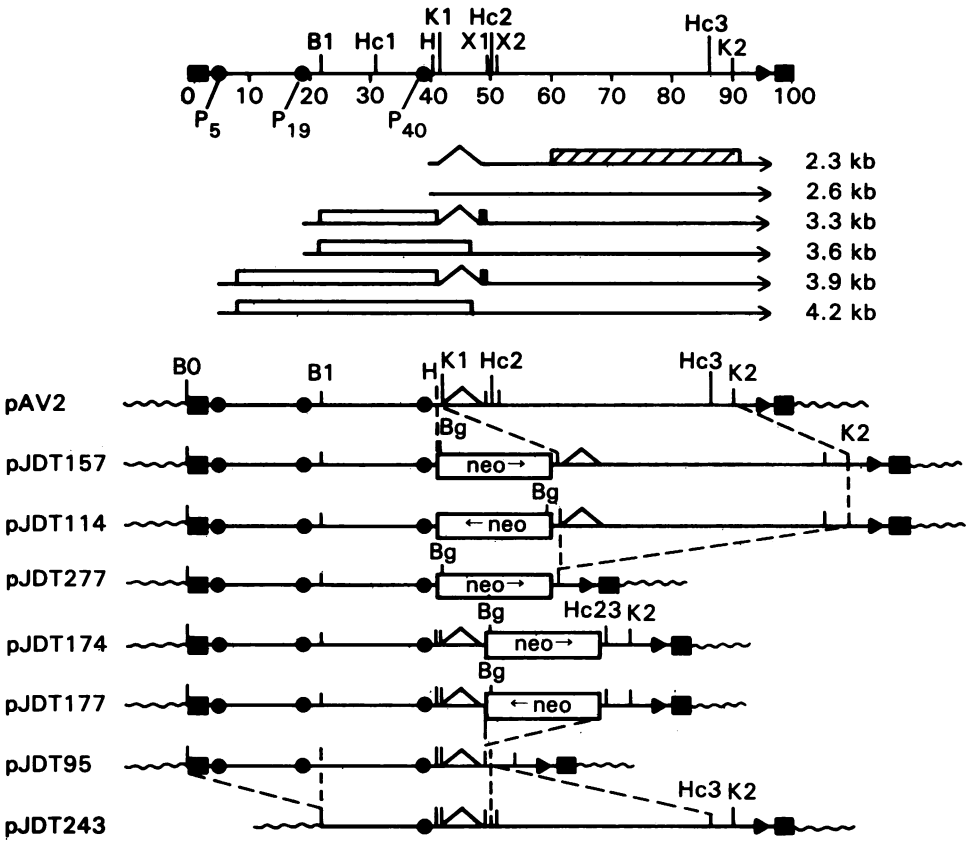

FIG. 2. Structure of the AAV genome and additional AAV recombinant plasmids and vectors. The structure of the AAV genome is shown in the top portion on a scale of 100 map units. The AAV genome is 4,675 nucleotides long (36, 44), and 1 map unit is approximately 47 nucleotides. Restriction endonuclease cleavage sites are indicated by the abbreviated nomenclature described in the legend to Fig. 1. Solid boxes show the terminal repeat sequences which make up the replication origins. Transcriptional control signals (7) are indicated as follows: promoters (solid circles), polyadenylation site (arrow head), intron (caret). The cytoplasmic RNA species (27, 31) are indicated by horizontal arrows. The major open reading frames are indicated by open boxes. orf-1 has at least four putative proteins depending on whether it is read from the spliced or unspliced p_5 or p_{19} transcripts. orf-2 is read from p_{40} transcripts and codes for the major capsid protein (see text). The lower portion shows the structure of AAV plasmids and vectors. All the constructs are derived from pAV2. For simplicity the recombinants are shown in linear form, and most of the plasmid is omitted. (Detailed structures of pJDT157 and pJDT277 are shown in Fig. 1.) The structures are drawn to the same scale as the AAV genome map, and the promoters, intron, and polyadenylation site are indicated as above. The DNA regions are indicated as follows: AAV (single line), *neo* coding sequence and 5′ flanking HSV sequences (open box), AAV terminal palindromes (solid box), and plasmid DNA (wavy line).

were generated by transfecting adenovirus-infected 293 cells with pJDT277 and a second AAV plasmid to provide AAV capsid protein. The complementing plasmid was either the wild type (wt), pAV2, or a replication-deficient mutant such as pJDT243. The 293 cells (4×10^6 cells per 60-mm-diameter dish) were infected with adenovirus type 2 (20 PFU per cell) and 1 h later were transfected with pJDT277 DNA (10 μg per dish) and the complementing plasmid pAV2 (10 μg) or pJDT243 (10 μg). After 48 h the cells were harvested, frozen and thawed three times, and heated at 60°C to inactivate the adenovirus. This lysate was then used in two alternative procedures as follows. (i) The cell lysate was passaged in a spinner culture of human KB cells (100 ml, 3×10^7 cells) together with helper adenovirus type 2 (10 PFU per cell) but in the absence of any added AAV particles, since the original lysate usually has sufficient wt AAV particles generated either from the pAV2 or from recombinant wt AAV (40). One-half the particles from this culture were used to infect a second spinner culture of KB cells (1 liter, 3×10^8 cells) together with fresh helper adenovirus. After 44 h, AAV particles were purified by banding once in CsCl equilibrium density gradients (6, 21). Particles banding between 1.39 and 1.36 g/cm^3 were taken, since these were expected to be enriched for AAV-*neo* genomes. This fractionation also removed adenovirus and many of the wt AAV particles. The preparation of particles was dialyzed at 4°C against Tris hydrochloride (pH 8.0)–0.3 M NaCl and then heated at 60°C to inactivate any residual adenovirus. (ii) Particles were taken directly from the plate lysate or after an additional cycle of amplification in a spinner culture of KB cells (as described above). In either case, AAV particles were then purified from cell lysates by sedimentation through glycerol onto a cushion of 60% (wt/vol) metrizamide (11).

Estimation of the titer of AAV-transducing particle preparations. The ratio of AAV-*neo* to wt genomes in particle preparations was determined by gel electrophoresis of aliquots of the DNA extracted from the particles or by infecting a portion of the lysate into 293 cells with helper adenovirus and measuring the ratio of duplex replicative-form (RF) DNA for wt and AAV-*neo* genomes in a Hirt lysate (45). The approximate number of AAV particles in purified preparations was estimated spectrophotometrically (9). In crude plate lysates the particle number was estimated by infecting a portion of the lysate into 293 cells with helper adenovirus

TABLE 2. Expression of *gen*^{r} phenotype and transfection with vector DNA

Expt no.	Cells	Vector DNA	Frequency of *gen*^{r} colonies[a] from cells plated at:	
			2.5×10^5 per dish	5×10^4 per dish
1	293	pJDT157	4.4×10^{-5}	ND
		pJDT157-BglII cleaved	1.0×10^{-6}	ND
		pAV2	0	ND
2	293	pJDT157	2.4×10^{-5}	1.2×10^{-4}
		pJDT114	0	0
		pJDT174	2.0×10^{-6}	1.4×10^{-5}
		pJDT177	0	0
		pAV2	0	0
3	293	pJDT277	ND	3.3×10^{-4}
		pAV2	ND	0
4	HeLa	pJDT277	3.0×10^{-4}	2.0×10^{-4}
		pAV2	0	0

[a] Cells grown in 35-mm-diameter dishes (2.5×10^6 cells per dish) were transfected with vector DNA (7.5 μg per dish). One day after transfection the cells were trypsinized, and 2.5×10^5 cells or 5×10^4 cells were replated into 75-cm^2 plastic T-flasks in fresh medium. Three days later cells were placed under selection by adding geneticin (400 μg of active component per ml for 293 cells or 600 μg of active component per ml for HeLa cells) to the medium. Colonies were counted after an additional 10 to 20 days.

and comparing the amount of duplex RF DNA in a Hirt lysate with that from control cells infected with known amounts of purified AAV particles (20, 45). Infectivity of purified AAV particles was determined by the indirect immunofluorescence assay (9).

Selection of *gen*^{r} cells. Approximately 10^6 cells growing in 35-mm-diameter dishes were infected with transducing particles or transfected with vector DNA. After 24 h the cells were trypsinized and replated at lower dilutions of 10^5 or 5×10^4 cells per 75-cm^2 T-flask in fresh medium. Three days later, the medium was supplemented with geneticin (GIBCO Laboratories) at 500 μg of active component per ml for 293 cells or 600 μg/ml for HeLa cells. Cultures were then maintained for 10 to 20 days until resistant colonies could be counted.

Some cultures were allowed to grow into mass cultures after the appearance of *gen*^{r} colonies. Individual clones were derived from mass cultures of *gen*^{r} cells by cloning at terminal dilution in 96-well microtiter dishes. Clones derived from wells which received only one cell were expanded in T-flasks. All cloning was performed in the absence of geneticin, but all clones were *gen*^{r} when subsequently tested in selective medium.

DNA extraction and analysis. Genomic DNA was extracted from cells (50) and cleaved with restriction endonucleases. Rescue of vector DNA was analyzed by infecting *gen*^{r} cells with adenovirus type 2 (20 PFU per cell) in the presence or absence of AAV2 particles (10 infectious units per cell). After 48 h, viral DNA was selectively extracted by a modified Hirt procedure (20, 46). Cleaved genomic DNA or DNA from Hirt lysates was analyzed by electrophoresis in 0.7% agarose gels, blotting to nitrocellulose membranes, and hybridizing with nick-translated ^{32}P-DNA probes.

RESULTS

Structure of AAV2 genome and vectors. The AAV2 genome structure is summarized in Fig. 2, which indicates the location of the three transcription promoters (p_5, p_{19}, and p_{40}), the 335-nucleotide intron, and the polyadenylation site(3, 7, 16, 27, 28, 32, 44). The AAV vectors described here were constructed to allow expression of the inserted *neo* gene under the control of the AAV p_{40} transcription unit. The *neo* coding sequence was obtained by excision of a DNA fragment from pSHL72 which contained a few 5′ nucleotides from the HSV TK gene but no transcriptional regulatory sequences (Fig. 1). In pJDT 157 the *neo* sequence was inserted at the *Hin*dIII site within the 55-nucleotide leader of the normal AAVp_{40} transcript and 26 nucleotides before the intron (16, 27), but in pJDT277 the intron was deleted. In both vectors, splicing of the chimeric transcript was not required to allow translational expression of *neo*. In pJDT174 the *neo* sequence is inserted downstream of the AAV intron, and splicing may be required to allow translation.

The reading frame from orf-1 codes for several putative *rep* proteins, one or more of which are required for AAV duplex (RF) DNA replication (19, 41, 46). Deletions or frameshift mutations to the left of the *Hin*dIII site prevent AAV RF DNA replication (*rep*$^-$). orf-2 codes for the major capsid protein VP3 (19, 46). Mutations in orf-2 or in the intron allow RF DNA synthesis (*rep*$^+$) but block capsid protein synthesis (*cap*$^-$) and accumulation of progeny single-strand DNA. All of the AAV-*neo* vectors shown here have a Rep$^+$ phenotype for AAV DNA replication, and when transfected into adenovirus-infected cells the AAV-vector genome is excised from the plasmid and replicated to generate duplex RF molecules (data not shown). However, the vectors are *cap*$^-$ and thus cannot produce infectious virus particles.

Selection of *gen*^{r} cells after transfection with vector DNA. When 293 cells were transfected with the AAV-*neo* vector pJDT157, *gen*^{r} colonies were obtained at a frequency of about 10^{-4} (Table 2). Insertion of the *neo* sequence in the opposite orientation from that in pJDT114 or cleavage of pJDT157 with *Bgl*II (which separates the *neo* sequence from the p_{40} promoter) decreased the frequency of *gen*^{r} colonies. Thus most of the *gen*^{r} colonies obtained with pJDT157 were in fact due to expression controlled by p_{40}. The vector pJDT157 and the intronless derivative pJDT277 gave similar frequencies of *gen*^{r} colonies. The plasmid pJDT174, in which the *neo* sequence was inserted downstream of the AAV intron, yielded about 10-fold fewer *gen*^{r} colonies than did pJDT157. When 293 cells were plated after transfection

TABLE 3. Transformation of cells to *gen*^{r} with AAV-transducing particles[a]

Expt no. and transducing virus preparation	Ratio of wt to *neo*[b]	No. of particles/ cell[b]	Total no. of AAV-*neo* particles[b]	*gen*^{r} colony frequency (%)			
				HeLa cells		293 cells	
				10^5	5×10^4	10^5	5×10^4
Expt 1							
(AAV/JDT277)	1:1	50,000	25,000	2.5	1.5		
		5,000	2,500	2.1	1.1		
		500	250	1.25	0.85		
		50	25	0.25	0.15		
		5	2.5	0.025	0.02		
		0.5	0.25	0.006	0.005		
Expt 2							
(AAV/JDT277)	5:1	1,000	200		0.47		0.27
		100	20		0.45		0.09
		10	2		0.1		0.02
		1	0.2		0.01		0.001
		0			0		
Expt 3							
AAV/JDT277	2:1	5,000	1,400	0.52	0.42	0.02	0.01
		1,000	330	0.06	0.06	0.04	0.01
JDT243/JDT277	1:1	5,000	2,500	0.68	0.48	0.01	0.03
		1,000	500	0.13	0.10	0.08	0.03
JDT243/JDT277 (1× passage)	1:1	10,000	5,000	1.6	1.4	0.03	0.07

[a] The virus preparations used in experiments 1 and 2 were prepared by method A and those in experiment 3 were prepared by method B as described in Materials and Methods. In experiment 3, two preparations were purified directly from plate lysates and one preparation as indicated was passaged once before purification. The preparations were grown by complementing pJDT277 with either pAV2 (AAV/JDT277) or pJDT243 (JDT243/JDT277).

[b] The number of particles and the ratio of wt to vector genomes was determined for each preparation as described in Materials and Methods. From these data, the number of AAV-*neo* particles was calculated. Note that MOIs are reported as particles per cell. For wt AAV, 1 infectious unit is equal to approximately 100 particles (6). For geneticin selection the cells were replated 24 h after infection as described in Materials and Methods at concentrations of 10^5 or 5×10^4 per 75-cm^2 T-flask as indicated.

(Table 2) at higher density (2×10^5 per dish), the frequency of *gen*^{r} cells was fivefold lower than for 293 cells plated at a lower density (5×10^4 per dish). The reason for the density dependence of *gen*^{r} is not known, but was seen in 293 cells at high but not low density and has also been reported for monkey CV1 cells (13). The density dependence was not seen for HeLa cells (Table 2) and was less obvious even for 293 cells after infection with transducing particles (see discussion of Table 3 below).

These results showed that the AAV promoter p_{40} could drive stable expression of a dominant, selective marker in mammalian cells. Since pJDT277 gave good expression and was small enough to package into AAV particles, it was used in further experiments.

Selection of *gen*^{r} cells after infection with AAV-*neo*-transducing particles. We previously showed that AAV vectors containing a foreign sequence (e.g., the procaryotic sequence for chloramphenicol acetyltransferase) could be packaged into AAV particles and that the gene could be transiently expressed when the particles were infected into cells in the presence of adenovirus (47). In the present study we tested whether introduction of the AAV vector into cells via infection with transducing particles in the absence of helper could increase the efficiency of expression and integration of a dominant selective marker. The vector JDT277 was packaged into AAV particles by complementing it with a *cap*$^+$ AAV plasmid (see Materials and Methods).

Cells were infected with transducing particles containing the AAV-*neo* vector JDT277 and then placed under selection for the *gen*^{r} phenotype (Table 3). Clones of *gen*^{r} HeLa cells were obtained at a frequency of up to 2 to 3%, which is much higher than that obtained after DNA transfection (cf. Table 2). In HeLa cells the transducing virus preparations yielded *gen*^{r} colonies in proportion to the number of particles containing AAV-*neo* vector genomes. However, in experiment 2 in Table 3 a plateau of about 1 to 2.5% was obtained at about 250 AAV-*neo* genomes per cell, and increasing the number of particles per cell by 100-fold did not increase the frequency. These data indicate that a single packaged vector genome is sufficient to allow transformation of cells to the *gen*^{r} phenotype. Comparison of the two transducing particle preparations in experiments 1 and 2 of Table 3 indicated that increasing the ratio of AAV-*neo* particles to AAV wt particles in the virus preparations by about fivefold did not significantly affect the frequency of *gen*^{r} clones. Table 3 also shows that the frequency of *gen*^{r} colonies was higher in HeLa cells than in 293 cells.

Rescue of the AAV-vector from *gen*^{r} cells. When pJDT277 DNA was transfected into adenovirus-infected cells the AAV-*neo* genome JDT277 could be rescued free of plasmid sequences and replicated to yield duplex RF molecules (data not shown). We also determined whether the JDT277 vector was rescuable from mass cultures of *gen*^{r} cells generated by infection with AAV-*neo* transducing particles (IN series) or transfection with pJDT277 (TR series) of 293 or HeLa cells. The cultures were placed under selection, and after the *gen*^{r} colonies appeared the culture flasks were maintained until the colonies developed into mass cultures. Thus, individual mass cultures arose from multiple colonies as indicated in Fig. 3. The individual mass cultures were then tested for rescue of the AAV-*neo* vector by superinfection with adenovirus or adenovirus together with AAV particles. Rescue of the vector was then scored at 48 h after helper virus infection by selective extraction in the Hirt lysis procedure,

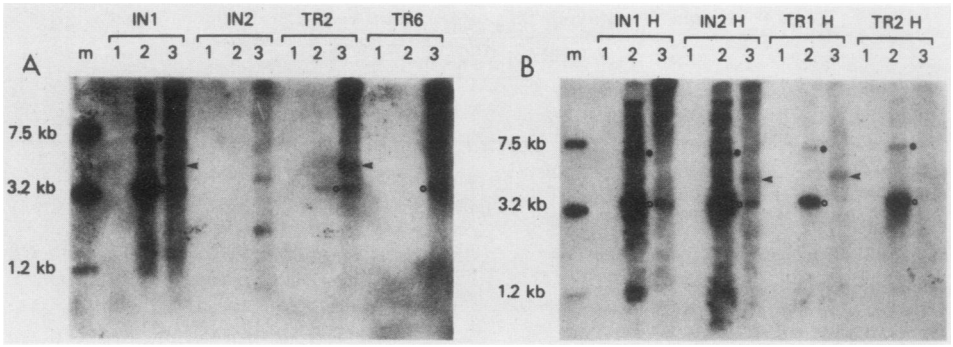

FIG. 3. Rescue of the AAV vector from cultures of *gen*^{r} cells. Mass cultures of (A) 293 or (B) HeLa cells were mock infected (track 1), infected with 15 PFU of adenovirus type 2 per cell (track 2), or adenovirus type 2 plus 10 IU of wt AAV2 per cell (track 3). Viral DNA was extracted 44 h later by the Hirt procedure and analyzed by agarose gel electrophoresis and blotting as described in the text. Each track represents the DNA isolated from 5×10^5 cells. The probe was ^{32}P-labeled *neo* DNA obtained by labeling a *Hin*dIII-*Bam*HI fragment obtained from within the *neo* coding sequence of the plasmid pNEO (P. L. Biochemicals). The position of the 3.3-kb vector DNA monomeric replicating form (○) and its 6.5-kb dimer (●) are indicated. The components marked (◀) probably arose by recombination between the AAV vector and wt AAV. Track m shows molecular weight markers generated by digesting pJDT277 with *Pvu*I or *Pvu*I plus *Sph*I and mixing fragments from both digestions. The mass cultures were derived from cultures flasks which originally contained the following number of *gen*^{r} colonies: IN1, 23; IN2, 24; TR2, 11; TR6, 12; IN1H, 41; IN2H, 75; TR1H, 30; TR2H, 43.

which is expected to extract free (unintegrated) vector genomes but not those which are covalently integrated into cell DNA. DNA from the Hirt extracts was electrophoresed in agarose, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled *neo* DNA probe. This resulted in detection of a 3.3-kilobase (kb) DNA species which is the expected size of the JDT277 vector. This 3.3-kb DNA was not observed in any cultures which had not been infected with adenovirus, but was observed in all HeLa cell mass cultures after adenovirus infection. The 6.5-kb species represents the expected dimeric replicating form of the 3.3-kb vector. The presence of this dimeric replicating form is characteristic of in vivo replicating AAV genomes (3, 19, 26, 38, 45, 46).

In the 293 cell mass cultures, the 3.3-kb vector was not rescuable with adenovirus alone in two cultures (IN2, TR6), and in the other two cultures (IN1, TR2) the rescue was less efficient than from similar HeLa cell IN or TR cultures. Since the 293 cell mass cultures arose from culture flasks which originally contained many independent colonies (Fig. 3), these observations may indicate that one or a few colonies were predominant in the resulting mass cultures.

The blotting shown in Fig. 3 was performed under conditions which could readily detect less than one vector genome per diploid cell equivalent. Therefore, these results show that in the mass cultures the vector probably was integrated into cellular chromosomes but could be rescued by providing helper functions. The experiment shown in Fig. 3 also showed that for HeLa cell mass cultures, rescue of the vector by adenovirus was inhibited in the presence of added wt AAV particles. This effect was regularly observed and is currently under study. It may simply represent competition for replication sites or replication proteins by the superinfecting AAV genomes.

In the presence of wt AAV there was a significant amount of a *neo*-containing species larger than the original 3.3-kb vector (Fig. 3). This apparently represents recombination between the rescued vector and the superinfecting wt AAV. A lesser amount of a similar type of component could be seen in the HeLa cell mass cultures IN1H and IN2H, but not in the TRH mass cultures, after rescue by adenovirus alone. This may reflect the presence of wt AAV genomes in the transducing particle preparations but not in the purified plasmid DNA used for transfection.

The AAV vector is integrated in the cell genome. No free copies of the AAV-*neo* vector were observed in the Hirt lysates of uninfected *gen*^{r} cells, which implied that all the vector genomes in these cells were integrated. This was examined further by restriction cleavage and blotting of genomic DNA isolated from *gen*^{r} cells as illustrated for several individual clones in Fig. 4. The IN series of clones were derived from 293 cells infected with transducing particles containing the vector JDT277, whereas the TR series were derived from 293 cells transfected with the vector DNA pJDT277.

The size of the vector JDT277 (which is separated from the plasmid DNA) when packaged into transducing virus particles is expected to be 3.3 kb. If the JDT277 genome existed as a free unintegrated episome in the IN series of clones, cleavage with *Bgl*II should yield a 1.55-kb *neo*-containing fragment if the genome were linear or a 3.3-kb fragment if the genome were circular or, more likely, oligomeric, as expected for a replicating AAV genome. In an analogous fashion, *Kpn*I cleavage would be expected to yield a 2.7- or 3.3-kb fragment. In fact for the IN clones (Fig. 4), both *Bgl*II and *Kpn*I cleavage gave fragments which when detected with a ^{32}P-*neo* probe were all larger than 3.3 kb. This supports the likelihood that in these cell lines the vector is integrated into the cell genome.

The plasmid vector pJDT277 has a size of 7.45 kb. Thus, in the TR series of clones, cleavage with *Kpn*I would yield a 7.45-kb *neo*-containing fragment if the vector remained unintegrated. For an integrated vector, the *Kpn*I *neo*-containing fragment would be of variable size, presumably at least 1 kb, which is the minimum size required to retain the *neo* transcriptional unit intact. In the TR series of clones (Fig. 4), *Kpn*I cleavage yielded a set of fragments which were of variable size, but in all cases were larger than 4 kb, and none were of the size (7.45 kb) expected for a free vector genome. In summary, these and additional restriction cleavages of genomic DNA from other *gen*^{r} 293 or HeLa cell clones support the conclusion that the AAV vectors are integrated into chromosomal sites in the *gen*^{r} cells. In the TR clones, whether the vector was integrated or free, *Bgl*II would be expected to generate a 1.55-kb *neo*-containing fragment, and this was observed in three of the clones (Fig. 4). One clone (TR2-3; Fig. 4) did not yield a 1.55-kb

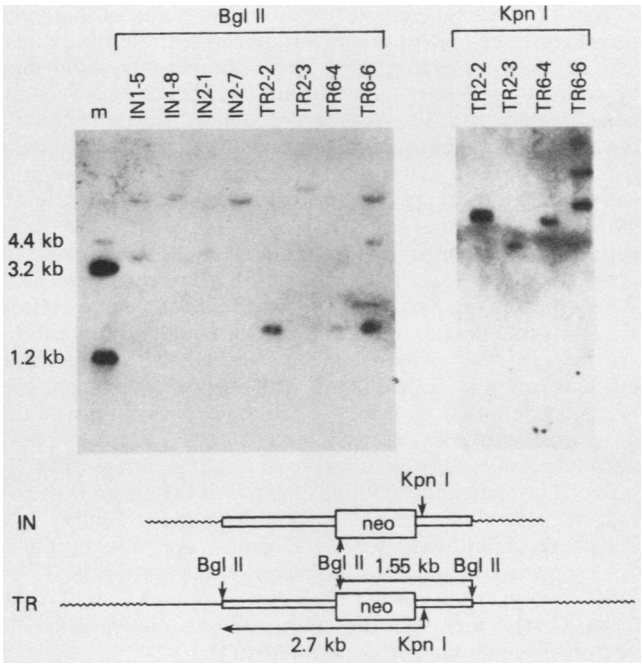

FIG. 4. Analysis of genomic DNA from *gen*^{r} 293 cells. Genomic DNA from individual clones of *gen*^{r} cells was cleaved with *Bgl*II or *Kpn*I, electrophoresed in agarose gels, and blotted to nitrocellulose. The hybridization probe (^{32}P-labeled *neo* DNA) and the marker fragments (shown on the left) were generated as described in the legend to Fig. 3. Individual clones are designated at the top. The IN series were derived by infection with transducing particles. The TR series were derived by transfection with DNA. The lower portion of the figure shows the relevant cleavage sites and the expected *neo*-specific fragments for the vector integrated into cell DNA, assuming there were no rearrangements or alterations in the AAV-*neo* region of the vector. Model IN shows the structures expected for *gen*^{r} cells derived from infection with transducing particles and model TR for clones from DNA transfection.

fragment, which suggests that in this clone the vector was rearranged such that the *Bgl*II site (site 2) was lost or the integration site was between the *neo* sequence and this *Bgl*II site.

The blotting data shown in Fig. 4 also provide some information on the copy number of the integrated AAV vectors. On the basis of calibration experiments (not shown), most of the *neo*-specific fragments shown in Fig. 4 are present at a low copy number per diploid cell genome. Thus, clones such as IN1-8, TR2-3, and TR6-4 may have a single gene copy of the vector. Other clones, e.g., IN1-5 and IN2-1, have at least two copies, and TR6-6 has at least three copies of the vector. Interestingly, the two clones TR2-2 and TR6-6 yielded a much higher molar amount of the 1.55-kb *Bgl*II fragment, which suggests that in these clones the vector may be present in multiple copies. This is also reflected in the *Kpn*I cleavage (Fig. 4), which for the clone TR2-2 shows a single band at about 6.5 kb. In this clone part or all of the vector is present in a higher copy number apparently as tandem series. Additional cleavage with other enzymes and blotting with an AAV ^{32}P-DNA probe (not shown) confirmed these conclusions. Similar results were also obtained with *gen*^{r} HeLa cell clones. These experiments also indicated that only very few of the IN clones contained integrated wt AAV, even though the transducing particle preparation contained wt AAV. Detailed mapping analysis of individual *gen*^{r} clones will be described elsewhere (M. G. Smith, manuscript in preparation).

DISCUSSION

We have shown that AAV is a highly efficient vector for introduction and stable expression of a selective marker into mammalian cells. In the particular experiments described here we used human cells, but the vector is also efficient for stable expression of *gen*^{r} in other cell lines such as mouse NIH 3T3 and for other selective genes such as *E. coli* xanthine-guanine phosphoribosyl transferase *gpt* (33), in human cells (M. G. Smith, unpublished data). Transfection of the vector DNA into cells resulted in expression of the selective marker at a frequency of about 10^{-4}, but this frequency was increased by at least 2 orders of magnitude by first packaging the vector into AAV-transducing particles. In either case all the *gen*^{r} clones contained the vector integrated at cellular chromosomal sites, and no free episomal (unintegrated) copies of the vector were detected. Most of the *gen*^{r} cells contained the vector present at low copy number. The vector could be rescued by infection of the cells with helper adenovirus together with AAV.

Hermonat and Muzyczka (18) also independently reported that AAV can be used as a transducing vector for high-frequency integration, expression, and rescue of a selective gene such as *neo*. Their results are similar to ours, except that in their vector the *neo* gene was expressed from an SV40 early gene transcription promoter. The two types of AAV vectors cannot be directly compared quantitatively because not only were different transcription promoters and different human cells lines used, but Hermonat and Muzyzcka (18) reported the number of vector particles added in terms of multiplicity of infection (6) rather than actual particle number. One multiplicity unit is equivalent to at least 100 particles for purified wt AAV particles and for preparations with variant AAV genomes (and perhaps for AAV-*neo* recombinants) it may be equivalent to many more particles (6). The SV40-driven AAV vector in human Detroit 6 cells gave a *gen*^{r} frequency of 1.3% for a multiplicity of infection of 100 and 3% for a multiplicity of infection of 1,000. Since these multiplicities are equivalent to at least 10,000 and 100,000 particles per cell, respectively, their data appear to be similar to ours in experiment 1 in Table 3. Both sets of data suggest that there may be some saturation level in the proportion of cells that stably express the *gen*^{r} phenotype. This could represent a host restriction, a limitation on the overall frequency of stable integration of the vector, the presence of many cryptic defectives in the transducing virus particles, or some inhibitory effect of the wt AAV particles which are also present. These issues remain to be resolved. However, when selective pressure was withheld until 7 days after infection of Detroit 6 cells with the SV40-driven AAV-*neo* vector at a multiplicity of 1,000, the frequency of *gen*^{r} cells was increased from 3 to 10%. Perhaps the apparent saturation level reflects in part the time required to obtain stable integration and expression of the *neo* gene.

In our experiments the transducing particles gave a higher frequency of *gen*^{r} on HeLa cells than on 293 cells. TheSV40-driven vector gave a sixfold-lower frequency in human KB cells than in Detroit 6 cells. It remains to be determined whether this represents differences in the stable integration rate between the two cell lines or differences in the control of AAV gene expression. Our previous studies suggest that, at least in transient assays after DNA transfection with AAV vectors, there are indeed differences in AAV gene expression between HeLa and 293 cells (47).

Whether the *gen*^{r} clones were derived from DNA transfection or transducing particles there were no major differences with respect to vector integration and copy number. Thus, the increased frequency of *gen*^{r} expression when the vector is used in transducing particles might reflect more efficient delivery of DNA to the cell via a virus particle. For instance, in the experiments shown in Table 2, transfection with 7.5 μg of vector DNA is equivalent to about 2×10^6 genomes per cell; this gave a transformation frequency of up to 3×10^{-4}. A transformation frequency 2 orders of magnitude higher than this was obtained with only 250 to 2,500 encapsidated vector genomes per cell. Thus, introduction of the *neo* gene in transducing particles is 3 to 4 orders of magnitude more efficient than by DNA transfection when considered on the basis of genomes per cell. In contrast to the wide difference in *gen*^{r} transformation efficiency between DNA transfection and transducing-particle infection, previous studies showed a much smaller difference with respect to AAV DNA replication in adenovirus-infected cells (26, 38). This could reflect differences in the mechanism or efficiency of integration dependent on the form of the AAV genome rather than differences in efficiency of delivery to the nucleus. In a study with a retrovirus vector, expression of the *neo* gene in mammalian cells was 100-fold higher when introduced in a transducing retrovirus virus rather than by DNA transfection (23). It was suggested that part of this difference was due to methylation of the DNA introduced by transfection. Whether this is the case for the AAV vector has not been determined.

Some aspects of the vector rescue remain unclear. The vector could be rescued from most of the mass cultures by infection with adenovirus alone. In contrast, for individual clones derived from such mass cultures, most were rescuable with adenovirus plus AAV, but less than 10% could be rescued by adenovirus alone (data not shown). Furthermore, in the rescue experiments (Fig. 3) there was a background of heterogeneous-sized (not 3.3 kb) DNA. While some of this represents replicating intermediates (3, 45), this material could also include defective or rearranged AAV-*neo* genomes generated during integration, passaging of cells, excision, or subsequent replication. Also, as noted above, we have not critically assessed the presence of variants or cryptic defectives in the transducing particle stocks. To maximize the usefulness of the AAV vector system it will be important to carefully control the number of defectives, since these can be generated rapidly in passaging of AAV stocks (6).

Because earlier work did not detect AAV gene expression in cells infected in the absence of helper virus or when the AAV genome was covalently integrated into cell chromosomes (22, 37), it was suggested that AAV transcription promoters might require an activating factor supplied by the helper or might be repressed by cellular factors (18). However, the present work and our previous study (47) shows that p_{40} has strong constitutive activity in the absence of any helper virus function in both a stably integrated state and a transient assay. Furthermore, p_{40} is active not only in 293 cells, which express the adenovirus E1A and E1B genes, but also in other human (HeLa), monkey (Vero, CV1), mouse (NIH 3T3), and hamster (BHK21) cell lines which contain no adenovirus genes (47; J.-D. Tratschin, M. West, B. Carter, M. Smith, and E. Katz, unpublished results). Thus, the AAV p_{40} promoter is a strong constitutive promoter in a variety of mammalian cell lines and may provide a very useful way to circumvent tissue-specific controls which otherwise exist for many viral and cellular gene promoters.

The AAV-transducing vector has a number of other useful properties. First, it provides an approach to study expression of genes either from a low-copy-number integrated state or from a high-copy-number unintegrated state. Second, genetic analysis of the AAV genome indicates that the only *cis*-acting sequence required for replication or for excision from recombinant plasmids (and presumably for rescue from cell chromosomes) are the terminal repeat sequences (19, 41, 46). Since both the AAV *rep* gene (orf-1) and the AAV capsid proteins (orf-2) can be supplied in *trans*, it should be possible to package at least 4.5 kb of foreign DNA in AAV-transducing particles. Of course, if the vector is used via DNA transfection there is no absolute upper limit on the size of the DNA which can be inserted. Third, the high efficiency of gene transduction with AAV suggests that this vector system may be useful for cloning genes simply on the basis of phenotypic expression from cDNA libraries packaged into AAV. Subsequent rescue would generate the gene probe. This may allow cloning of genes in the absence of any nucleic acid probe or sequence information. Fourth, the AAV vector has very few viral genes, and integration of AAV appears to have no biological effect on the host cell (22). Finally, AAV has not been associated with any known disease (14). Thus AAV may ultimately be a useful and safe vector for gene therapy experiments (1).

At present, other viral transducing vectors with comparable properties are those based on SV40, adenovirus, or retroviruses. The SV40 vectors can only be rescued generally by fusion with a permissive T-antigen-producing cell line such as COS (monkey) cells (4). Adenoviruses have been used as transducing vectors (25, 48), but construction and preparation of virus stocks containing foreign genes is laborious and is complicated by the presence of a large number of adenovirus genes. Also, integrated adenoviruses cannot be readily rescued. AAV can replicate in human, simian, and rodent cells and thus should be rescuable from a wide range of cells, provided the appropriate helper virus is used (5). Retrovirus vectors can also be rescued from a wide variety of cells and potentially can package larger inserts than AAV (8, 30, 32, 42).

In summary, AAV represents a new viral transducing vector for high-efficiency expression of genes in mammalian cells by using either AAV or other transcription promoters. One possible complication of the vector at present is the presence of wt AAV particles in the transducing preparations. These can be reduced by use of mutant AAV genomes for complementing vectors during packaging and by subsequent purification of the vector particles. In any case, it should be possible to construct cell lines that can provide AAV capsid proteins and perhaps the AAV *rep* protein as well, which would then allow growth of pure populations of transducing particles as has been achieved for retroviruses (8, 30). The other current questions with respect to the failure to transform all the cells in the population, the generation of defectives, and the inability to rescue from all *gen*^{r} clones have been mentioned above. Nevertheless, the present results do suggest that AAV is a very promising system to be exploited as a transducing vector, and this is important for increasing the spectrum of useful vectors available for manipulation of genes in mammalian cells.

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