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Adeno-Associated Virus General Transduction Vectors: Analysis of Proviral Structures

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We used two kinds of adeno-associated virus (AAV) vectors to transduce the neomycin resistance gene into human cells. The first of these (*dl*52-91) retains the AAV *rep* genes; the second (*dl*3-94) retains only the AAV terminal repeats and the AAV polyadenylation signal (428 base pairs). Both vectors could be packaged into AAV virions and produced proviral structures that were essentially the same. Thus, the AAV sequences that are required in *cis* for packaging (*pac*), integration (*int*), rescue (*res*), and replication (*ori*) of viral DNA are located within a 284-base-pair sequence that includes the terminal repeat. Most of the G418^r cell lines (73%) contained proviruses which could be rescued (Res$^+$) when the cells were superinfected with the appropriate helper viruses. Some produced high yields of viral DNA; others rescued at a 50-fold lower level. Most of the lines that were Res$^+$ (79%) contained a tandem repeat of the AAV genome (2 to 20 copies) which was integrated randomly with respect to cellular DNA. Junctions between two consecutive AAV copies in a tandem array contained either one or two copies of the AAV terminal palindrome. Junctions between AAV and cellular sequences occurred predominantly at or within the AAV terminal repeat, but in some cases at internal AAV sequences. Two lines were seen that contained free episomal copies of AAV DNA. Res$^+$ clones contained deleted proviruses or tandem repeats of a deleted genome. Occasionally, flanking cellular DNA was also amplified. There was no superinfection inhibition of AAV DNA integration. Our results suggest that AAV sequences are amplified by DNA replication either before or after integration and that the mechanism of replication is different from the one used during AAV lytic infections. In addition, we have described a new AAV general transduction vector, *dl*3-94, which provides the maximum amount of room for insertion of foreign DNA and integrates at a high frequency (80%).

Adeno-associated virus (AAV) is a human virus (7) which contains a single-stranded DNA genome approximately 5 kilobases (kb) in length (4, 6, 42). The virus can be propagated either as an integrated provirus or by lytic infection (1, 20, 21). Except under special circumstances (38, 47), AAV requires the presence of a helper virus for a productive infection; the helper virus can be a member of either the herpesvirus or adenovirus family (1, 10, 32, 35). When no helper virus is available, AAV is capable of establishing a latent infection in which the viral genome exists as an integrated provirus in the host cell (3, 5, 11, 15, 21). If a latently infected cell line is later superinfected with a helper virus, the AAV provirus is excised and proceeds through a normal productive infection.

The viral genome consists of a linear DNA molecule with inverted terminal repeats (145 base pairs [bp]). The first 125 bp of the terminal repeat is a complex palindrome which can exist in either of two orientations (30). Genetic analysis of AAV has revealed at least four regions of the genome with distinct phenotypes (see Fig. 1). The terminal repeats are the origins for lytic DNA replication (16, 37, 39) and appear to initiate DNA synthesis by a hairpin priming mechanism (29, 43). The first 15 to 20 bp of the terminal repeat has also been shown to facilitate excision of AAV DNA from recombinant plasmids (J. Gottlieb and N. Muzyczka, Mol. Cell. Biol., in press). The *lip* and *cap* regions code for the viral capsid proteins (2, 17, 22, 23, 42, 44) and are synthesized from transcripts that are initiated by a promoter at map position 40 (12–14, 28, 42). The *rep* region codes for a family of four nonstructural proteins (33, 42) which are required for lytic DNA replication (17, 44) and for the control of viral gene expression (24, 25, 46). The Rep proteins are coded for by a family of four transcripts synthesized from the p5 and p19 promoters (28, 31). All AAV transcripts appear to terminate at a polyadenylation signal at map unit 95 (42).

Although a substantial amount is known about AAV DNA replication and the genetic organization of the virus, relatively little is known about AAV integration. Previous studies of AAV latent infection have focused primarily on cells containing wild-type (wt) proviruses. Because AAV contains no selectable markers, these studies have relied on cell lines that (of necessity) were selected on the basis of containing an intact provirus that was capable of being rescued (3, 5, 11, 15, 20, 26). Genomic hybridization studies of these cell lines (3, 11, 26) indicated that they did, indeed, contain intact viral DNA, but it was not clear whether the absence of rearrangements was typical of all cells that might contain AAV sequences. In addition, these studies indicated that AAV DNA had integrated into host chromosomes in random positions with respect to cellular sequences and that the provirus usually occurred in the form of a tandem concatemer. It was not known which AAV sequences were essential for integration and rescue, whether all latently infected cells that contained an intact provirus could be rescued, or whether any AAV gene product influenced the frequency of integration.

We have previously suggested that the properties of AAV might make it useful as a general transduction vector. We also have shown that a selectable marker, when substituted for the viral capsid genes, could be introduced into human cells by AAV-mediated transduction (18). In this report, we compare the transduction and rescue frequencies of two

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kinds of AAV vectors: *dl*52-91 (17), which retains the AAV *rep* gene, and *dl*3-94, in which all of the AAV coding sequences have been deleted. Our results suggest that the AAV *rep* gene inhibits viral transduction, and our work with *dl*3-94 defines in part the essential sequences for viral integration and rescue, as well as the sequences required for packaging of viral DNA. We have also examined in detail the proviral structures that are generated by AAV vectors.

MATERIALS AND METHODS

Construction of recombinant plasmids. The construction of the vector *dl*52-91 and the recombinant *dl*52-91/neo have been described (17, 18). The vector *dl*3-94 was constructed by ligating a *Bgl*II-*Eco*RV double digest of the two plasmids *dl*3-49 and *dl*49-94. *dl*49-94 has been described previously (17). *dl*3-49 was constructed by partial digestion of pSM620 (36) with *Bst*NI to remove AAV sequences between map units 3 and 49 followed by the insertion of a *Bgl*II linker at the site of the deletion. Because of the close proximity of the palindromic AAV termini in *dl*3-94, the AAV ends were unstable in *Escherichia coli* HB101 and were rapidly deleted. To maintain the integrity of the AAV termini, we constructed a variant of *dl*3-94 which contained a stuffer insert that separated the ends of AAV. The variant was called *dl*3-94I_2 and was constructed by ligating a *Kpn*I-*Eco*RV double digest of the two plasmids *dl*3-49 and *dl*49-94. This produced a plasmid which contained a 2.3-kb stuffer insert consisting of internal AAV sequences. *Bgl*II digestion removes the stuffer insert, leaving the *dl*3-94 AAV deletion. We have subsequently discovered that *dl*3-94 can be maintained stably in *E. coli* JC8111 (8) without a stuffer fragment. We thank Peter Tattersall, Yale University, for kindly providing JC8111.

*dl*3-94/neo was constructed by ligating a *Bam*HI digest of pHM2609 (18) to a *Bgl*II digest of *dl*3-94I_2. (The plasmid pHM2609 contains the simian virus 40 (SV40)-neo cassette on a *Bam*HI fragment.) The resulting *dl*3-94/neo plasmid was stable in *E. coli* HB101 (9).

Cells and viruses. Human Detroit 6 (D6), B7374IIID5 (hereafter referred to as D5), KB, and 293 cells were grown in monolayer cultures. D5 cells were obtained from K. I. Berns, Cornell Medical School, and are a human D6 cell line which is latently infected with wild-type AAV (11). Stocks of adenovirus type 2 were produced in HeLa suspension cells, and titers were determined by plaque formation on monolayer cultures of HeLa or KB cells. Recombinant AAV virus stocks were produced as previously described (18). Titers of *dl*52-91/neo and *dl*3-94/neo were found as previously described, except that wt AAV (multiplicity of infection [MOI], 5) was added when the titer of *dl*3-94 was being measured.

Titer of recombinant viral stocks. The titer of the *dl*52-91/neo stock was previously described (18). The method for determining the wt virus contamination in both the *dl*52-91/neo and the *dl*3-94/neo stocks was as previously described (18). The titer of the *dl*3-94/neo stock was measured in two ways. The first method was a modification of that described previously (18). *dl*3-94/neo virus (100 or 200 μl), wt AAV (MOI, 2), and adenovirus (MOI, 2) were used together to infect D6 cells, the Hirt DNA was isolated, and the amount of replicating *dl*3-94/neo DNA was determined by Southern blotting. Control experiments were done in which various amounts of wt AAV (10^3 to 10^5 total virus particles) plus adenovirus (MOI, 2) were used to infect cells. By comparing the amount of replicating *dl*3-94/neo DNA produced by an unknown amount of virus with the amount of replicating wt AAV produced by a known amount of virus, we estimated the recombinant virus titer to be approximately 10^4 virus particles per ml.

In addition, the titer of the *dl*3-94/neo stock was measured by a modification of the method used by Yakobson et al. (47). Briefly, approximately 30,000 D6 cells in a microdilution plate were infected with 10 or 100 μl of *dl*3-94/neo virus stock, along with wt AAV and adenovirus. After 30 h, the cells were trypsinized and suspended in 5 ml of phosphate-buffered saline. Various dilutions of the cells were trapped on nitrocellulose by filtration under suction. The filters were then treated in the same way as described by Yakobson et al. (47). The titer of *dl*3-94/neo by this method was also found to be 10^4 infectious units per ml.

The particle titers of recombinant and wt stocks used in this study were also determined by using a modification of the dot-blotting technique. Briefly, 0.01 to 0.05 ml of a virus stock was treated with pancreatic DNase (50 μg/ml of crude virus lysate) for 1 h at 37°C. The DNase was then inactivated by heating at 95°C for 15 min. The mixture was treated with proteinase K (C_f = 1 mg/ml) at 37°C for 1 h. After a phenol extraction, the mixture was denatured with NaOH (C_f = 0.2 N) and boiled for 10 min. Then 10 volumes of cold 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were added, and the mixture was filtered through nitrocellulose by using a Schleicher & Schuell Minifold apparatus. The filter was baked at 80°C for 2 h, and then hybridized to AAV or pNeo [^{32}P]DNA. The dot blots were analyzed by using the Ambis Scanning System II from Automated Microbiology Systems. The particle-to-infectivity ratio of the wt and *dl*52-91/neo stocks was found to be 10 to 20:1. The particle titer of the *dl*3-94/neo stock was below the sensitivity of this assay, and we concluded that the particle-to-infectivity ratio was less than 100:1.

Transduction with recombinant viral stocks. *dl*52-91/neo virus at various multiplicities was used to infect 10^2 to 10^5 D6, D5, or KB cells in 96-well microdilution plates. The day after infection, the cells were split into 10-cm dishes, and G418 (C_f = 1 mg/ml) selection was begun at various times from 12 h up to 1 week postinfection. Approximately 1 to 2 weeks after the addition of G418, resistant colonies were counted and picked. In all, 42 individual G418 clones were picked, each from a separate dish. Eight clones were picked from D6 cells infected at an MOI of 0.1 and have a name beginning with the letter H (H series). The C series consists of 15 clones picked from D6 cells infected at an MOI of 1, 14 KB clones (2 at an MOI of 1 and 12 at an MOI of 10), and 5 D5 clones at an MOI of 10.

*dl*3-94/neo virus was used to infect D6 cells at an MOI of 0.03. The virus was allowed to adsorb to the cells overnight, and the next day the cells were split into 10-cm dishes. Approximately 40 to 48 h later, G418 at a final concentration of 1 mg/ml was added to the cells. Approximately 1 week after G418 addition, resistant colonies were counted and 19 clones were picked for further analysis (S series).

Genomic hybridizations. Genomic DNA was isolated as previously described (34) from 42 G418^r clones and digested with the appropriate restriction enzymes, electrophoresed on 1.0 or 1.4% agarose gels, transferred to a nitrocellulose filter (40), and hybridized to ^{32}P-labeled nick-translated probes as previously described (34). *dl*52-91/neo plasmid DNA was the probe for clones that had been transduced with *dl*52-91/neo virus. For clones that had been transduced with *dl*3-94/neo virus, the probe was pNeo (41), which contains

only the procaryotic neomycin sequences present in *dl*3-94/neo.

Rescue assays. Each of the transformed cell lines was tested for the ability of the AAV recombinant provirus to be rescued in the presence of adenovirus. Adenovirus type 2 at an MOI of 2 was added to the cell line; for cell lines transformed with *dl*3-94/neo virus, AAV was also added (to provide *rep* functions) at an MOI of 1. At 40 to 48 h postinfection, low-molecular-weight DNA was isolated by the method of Hirt (19), fractionated on a 1.4% agarose gel, transferred to a nitrocellulose filter (40), and probed with ^{32}P-labeled pSM620, *dl*52-91/neo, or pNeo-specific probe. Rescue of the provirus was indicated by the presence of a *dl*52-91/neo or a *dl*3-94/neo band of monomer, duplex size. The identity of the band was confirmed by restriction endonuclease digestion.

Transduction frequencies were calculated as previously described (18) and represented the average of five experiments. The transduction frequency determined from low-MOI infections took into account the number of viral infectious units (as determined by the titer) which were actually being used to infect the cells. The frequency was calculated as the number of transductants per infectious unit × 100. For example, at an MOI of 0.03 on 10,000 cells, only 300 infectious virus units are being added to the cells, and 300 G418^r colonies would then represent a transduction frequency of 100%. The number of double hits under these conditions was considered negligible. For *dl*3-94/neo, five individual experiments were done in which 0.10 ml of virus stock (10^4 infectious units/ml) was used to infect approximately 32,000 D6 cells in a microdilution plate. On day 1 after infection the cells were transferred into 10-cm dishes, and G418 selection was applied on day 3. The number of G418^r colonies ranged from 656 to 1,027 per experiment. *dl*52-91/neo transduction frequencies were determined in a similar way, and the frequencies obtained confirmed those reported previously (18). The 61 individual clones were tested for their ability to be rescued, and 42 were further analyzed by genomic hybridization. After being picked, the clones were maintained in the absence of G418. No significant loss of G418 resistance was seen up to 100 passages after isolation of the clones.

RESULTS

Location of AAV packaging sequence within a 280-bp sequence that includes the terminal repeats. The two recombinant AAV clones used in this study are illustrated in Fig. 1. *dl*52-91/neo has been previously described (18). It contains the AAV *rep* genes which are required for autonomous replication (17, 44) and for the control of AAV gene expression (24, 25, 46). *dl*3-94/neo contains none of the known AAV coding regions. Both constructs contain an identical foreign DNA insert which consists of the neomycin resistance gene downstream from the SV40 enhancer-promoter region.

*dl*3-94 (Fig. 1) is one of a family of general-purpose AAV transduction vectors that we have constructed. It consists of the terminal repeats of AAV (145 bp each) and an additional 139 bp of AAV sequence from the right-hand end of the genome which was retained because it contains the AAV polyadenylation signal. In addition, *dl*3-94 contains two unique restriction sites (*Bgl*II and *Nco*I) which are available for the insertion of foreign DNA. A number of variants of *dl*3-94 have been constructed which differ only in the restriction sites that are available for cloning (not shown). In

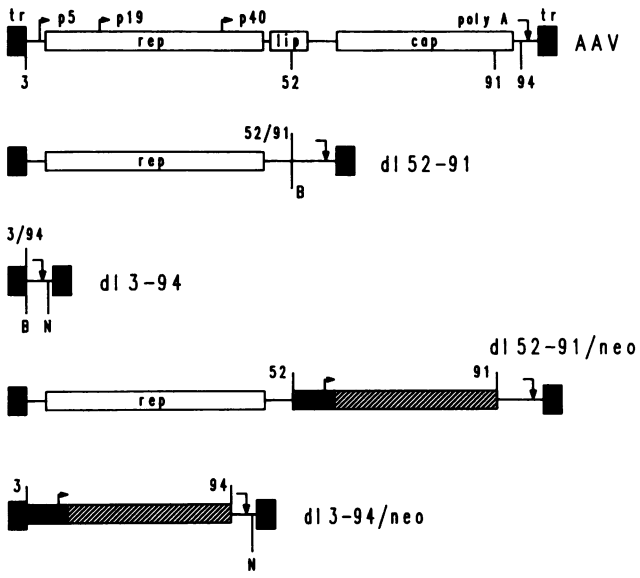

FIG. 1. Structure of AAV recombinant clones. The wt AAV is described in the text. Symbols: ▤, AAV terminal repeats (tr); ↴, AAV polyadenylation site (poly A) at map unit 95 (bp 4425); ↱ at map units 5 (bp 255), 19 (bp 843), and 40 (bp 1822), locations of the three AAV promoters; |, site of the deletion in each vector (*dl*52-91 has a deletion from map unit 52 [bp 2416] to map unit 91 [bp 4264]; *dl*3-94 contains a deletion from map unit 3 [bp 144] to 94 [bp 4397]). The two AAV recombinant clones contain the SV40 enhancer (▭) and the neomycin phosphotransferase gene (▨) inserted in each vector at the site of the deletion. The unique restriction sites in each vector, *Bgl*II (B) and *Nco*I (N), are also indicated.

*dl*3-94/neo, the SV40-neo cassette was inserted into the *Bgl*II site just downstream of the left-hand AAV terminal repeat.

Both *dl*52-91/neo and *dl*3-94/neo were successfully used to produce viral stocks by complementation with a recombinant AAV genome, *ins*96/λ-M (18), which supplied the missing AAV gene products in *trans* but was itself too large to be packaged. The *dl*52-91/neo stock had been previously characterized and was found to contain 10^6 infectious units/ml, of which approximately 20% consisted of wt virions (18). The *dl*3-94/neo virus stock was characterized in a similar way and was found to have a titer of 10^4 infectious units/ml, of which approximately 10% was wt virus. The fact that *dl*3-94/neo could be packaged means that the AAV sequences present in this construct are sufficient for the packaging of virion DNA. Thus, the AAV packaging sequence must be either in the 145-bp terminal repeats or in 139 bp (nucleotides 4397 to 4536) of internal AAV DNA. The wt contamination in both stocks is presumably the result of homologous recombination between the complementing AAV genome, *ins*96/λ-M, and the *neo* clones. In both cases there was either 87 or 226 bp of homology between the *ins*96/λ-M complementing plasmid and the *neo* constructs that could generate a wt genome.

More efficient transduction of mammalian tissue culture cells by *rep* clones than by *rep*$^+$ clones. Both viral stocks were used to infect either human D6 or KB cells, and G418^r colonies were either counted or picked and expanded for further study at 1 to 3 weeks after G418 selection was applied. To determine the relative transduction frequencies of the *rep*$^+$ and *rep* clones, we infected D6 cells with the two viral stocks at low MOIs (0.03 to 0.1). Low MOIs were used in this experiment primarily to minimize the possible effect of coinfection by the wt virus contaminant on the transduc-

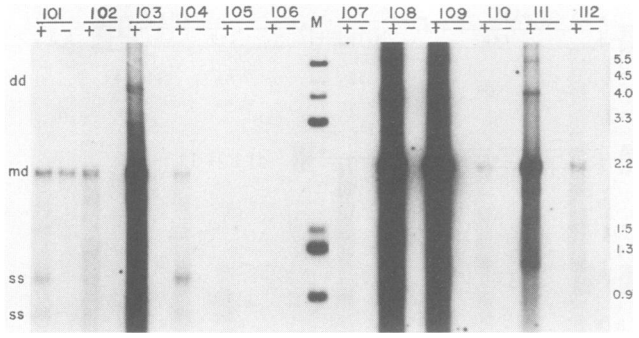

FIG. 2. Rescue of *dl*3-94/neo transduced cell lines. Rescue analysis was done on several *dl*3-94/neo-transduced cell lines as described in Materials and Methods. Low-molecular-weight DNA from the indicated S clone was electrophoresed on a 1.4% agarose gel, blotted, and probed with ^{32}P-labeled pBRNeo probe. The DNA in lanes labeled (+) was isolated from cells superinfected with both wt AAV and adenovirus type 2; DNA in lanes labeled (−) was isolated from cells infected with adenovirus alone. The positions of dimer duplex (dd), monomer duplex (md), and single-stranded (ss) *dl*3-94/neo, along with the sizes of DNA marker fragments, are indicated on the sides of the gel. The two single-stranded bands are believed to be different conformations of monomer-length single-stranded DNA.

tion frequency, and the frequency was calculated as the number of G418^r colonies per infectious AAV unit (see Materials and Methods). The results suggested that there was a substantial difference in the transduction frequencies of the two vectors. As previously reported (18), the transduction frequency of *rep*$^+$ *dl*52-91/neo was 0.4%, whereas that of *dl*3-94/neo was 80%. To see whether there were any other significant differences between *rep*$^+$ and *rep* viral transducing particles, we isolated a total of 61 individual G418^r colonies and examined them by several criteria.

Ability of most AAV proviruses to be rescued. AAV proviruses can be rescued when cell lines carrying a provirus are superinfected with a helper virus. To determine the percentage of G418^r cell lines which could be rescued, we infected each line with the appropriate helper viruses. Cell lines that had been transduced with *dl*52-91/neo were infected with adenovirus; cells that had originally been infected with the *rep dl*3-94/neo transducing virus were infected with adenovirus and wt AAV. Figure 2 illustrates our results with 12 randomly chosen *dl*3-94/neo proviral cell lines, and Tables 1 and 2 summarize the results for all of the lines that were examined. Most of the G418^r clones (73%) could be rescued and therefore contained at least one complete copy of the recombinant genome. Some cell lines were rescued quite efficiently to produce viral DNA yields that were comparable to those seen when cells were infected exogenously with virus (Fig. 2, S103, S108, S109, and S111). Other lines produced yields of DNA that were 50- to 100-fold lower. These two types of proviral lines were arbitrarily called high- and low-rescue clones, respectively (Tables 1 and 2). The products of rescue were the expected mixture of monomer duplex linear DNA (which is the major AAV replicative form), concatemeric duplex molecules, and single-stranded progeny DNA (Fig. 2). A few low-rescue lines (Fig. 2, S107; Table 2, S114 and C2D1) contained proviruses that were shorter than full length but were still capable of rescue. The remaining cell lines (20 to 25%) were incapable of rescue. Finally, when *rep*$^+$ and *rep* proviral lines were compared (Table 1), there was little difference in the frequency of rescue (68 versus 84%). The small difference that was observed appears to be due to differences in the time at which G418 selection was applied.

Time of selection. We noticed that most of the rescue-negative (Res$^-$) clones (Table 2) were the result of infections in which G418 selection had been applied within the first 24 h after infection. We investigated the effect of time of selection on 59 clones. Of 14 clones undergoing selection on day 1, 6 (43%) were Res$^+$, whereas of 45 clones undergoing selection on days 2 to 7, 37 (82%) were Res$^+$. Clones undergoing selection on day 1 were the result of infections with *dl*52-91/neo virus; clones undergoing selection on days 2 to 7 were obtained with either *dl*52-91/neo or *dl*3-94/neo virus; all but one of the clones used D6 cells as the parental line; the exception was a KB clone. These results suggest that delaying G418 selection for 48 h after infection increases the chances of isolating a rescue-positive (Res$^+$) clone. In addition, we mentioned above that there was a slight difference in the frequency of rescue of *rep*$^+$ and *rep* transductants (Table 1). All of the transductants selected on day 1 were *rep*$^+$ (Table 2). If these are eliminated from consideration, there is no difference in the frequency of rescue between *rep*$^+$ and *rep* proviruses (81 versus 84%).

Physical structure of AAV proviruses. To determine the physical structure of the recombinant proviruses, we examined a representative number of Res$^+$ and Res$^-$ G418^r clones by genomic hybridization (40). In all, 42 of the clones were analyzed. In each case, the genomic DNA was digested with restriction enzymes that cut once, more than once, or not at all within the proviral genome and hybridized to the appropriate probe. To determine whether the cellular AAV junction occurred at the terminal repeats of AAV, we also digested the DNA with enzymes that cut near the terminal repeats of the proviral genome. Finally, in each case, the intensities of digested bands were compared with those of known standards or with those of clones which were known to contain only one copy of the provirus to determine the copy number for each clone. The results are summarized in Table 2, and representative examples of the genomic hybridizations are shown in Fig. 3 to 5. Our observations of AAV proviral structures can be summarized as follows.

Presence of tandem repeats of the recombinant AAV genome in nearly all of the Res$^+$ clones. If the provirus were integrated in the form of tandem repeats, we would expect to see a genome-sized fragment when cellular DNA was cut with a restriction enzyme that cuts once within the recombinant genome. When *dl*52-91/neo transductants were cut with *Bgl*II or *Bam*HI, most of them produced a genome-sized band of 4.7 kb (Fig. 3A and 4). Figure 5 illustrates the pattern for several *dl*3-94/neo transductants that were cut with *Bgl*II to produce a common fragment of 2.3 kb. Of 28 Res$^+$ clones (Tables 2 and 3), only four clones apparently contained an integrated, single-copy provirus that could be rescued (S112 to S115). Because of the limitations of genomic blotting, we could not be certain that these clones did

TABLE 1. Rescue of recombinant proviruses

Recombinant virus	No. (%) of clones rescued			Total no. rescued/total no. (%)
	High	Low	None	
*dl*52-91/neo	11 (28%)	16 (40%)	13 (33%)	27/40 (68%)
*dl*3-94/neo	6 (32%)	10 (53%)	3 (16%)	16/19 (84%)
Total	17 (29%)	26 (44%)	16 (27%)	43/59 (73%)

TABLE 2. Summary of genomic data

Clone[a]	Complete genome[b]	Copy no.[c]	No. of integration sites[d]	Tandems[e]	Rescue	Selection day[f]	Type[g]
HN21	+	20	1	+	High	1	C2
S102[h,i]	+	15	2	+ (1)	Low	3	C1, A3
S101[j]	+	10	4	+ (2)	Low	3	
C10D7 (10/7)	+	10	1	+	High	7	C2
C10D6 (10/6)	+	10	1	+	High	6	C1
S108	+	10	1	+ (1)	High	3	
C105	+	7	1	+	High	1	C2
S116	+	6	2	+ (1)	High	3	
S118[j]	+	6	1	+ (2)	High	3	
C2D5 (2/5)	+	5	2	+	High	5	C1
S103	+	5	2	+ (1)	High	3	
S110	+	5	1	+ (2)	Low	3	
S111	+	4	1	+	High	3	
C10D5 (10/5)	+	7	2	+	High	5	C1
C6KB10	+	3	1	+	High	3	
C103	+	3	1	+	High	1	C1
S109	+	3	1	+ (1)	High	3	
C2D7 (2/7)	+	2	1	+	Low	7	C1
C2D2 (2/2)	+	2	1	+	Low	2	
S104	+	2	1	+ (1)	Low	3	
HN51	+	1.5	1	+	Low	1	C2
S112	+	1	1	−	Low	3	C3
S113	+	1	1	−	Low	3	
S115	+	1	1	−	Low	3	C3
S114[k]	−	1	1	−	Low	3	
S117	ND[l]	2	1	+	Low	3	
C10D2 (10/2)[m]	+	3	1	+	Low	2	C1
C10D3 (10/3)[m]	+	2	1	+	ND	3	C1
C2D1 (2/1)[k]	−	4	2	+	Low	1	A1
S107[k]	−	2	1	+	Low	3	A1
HN65[h,i]	−	10	1	−	Negative	1	A4
HN41[h,i]	−	6	1	−	Negative	1	A3
C102	−	6	1	−	Negative	1	A1
HN14[h,i]	−	6	1	−	Negative	1	A4
C101[h]	−	4	2	−	Negative	1	
HN61[h]	−	2	2	−	Negative	1	A3
S119	ND	2	1	−	Negative	3	
HN42[h]	−	<1	1	−	Negative	1	A2
HN22	−	<1	1	−	Negative	1	A2
C104	−	<1	1	−	Negative	1	A2
S105	−	<1	1	−	Negative	3	A2
S106	−	<1	1	−	Negative	3	A2

[a] Clones beginning with H and C were transduced with *dl*52-91/neo; S clones were transduced with *dl*3-94/neo. H clones were transduced at an MOI of 0.1; C clones were transduced at an MOI of 1, except for C6KB10, which was transduced at an MOI of 10; S clones were transduced at an MOI of 0.03. All of the clones shown in Table 2, with the exception of C6KB10, were derived from D6 cells.

[b] A clone was considered to have one full-length copy if it contained a genome-length fragment when digested with a one-cut enzyme or intact internal fragments when digested with enzymes which cut in the terminal repeats, i.e., *Sma*I, *Bal*I, or *Bgl*I.

[c] The copy number was estimated by comparing band intensities on the same gel with those of a clone which by restriction analysis contained only one copy. At least two different kinds of digests were used to estimate each clone.

[d] The number of independent integration sites per clone was estimated by counting the number of fragments produced by restriction enzymes which did not cut the proviral genome or cut only once.

[e] Clones contained a full-length tandem if they produced a genome-sized fragment when cleaved by a restriction enzyme that cut only once. Two clones (C2D1 and S107) containing a tandem repeat of a deleted *res*$^+$ genome were also classified as having full-length tandems. Numbers in parentheses indicate whether the proviral concatemer contained one or two copies of the terminal repeat at each AAV-AAV junction.

[f] Number of days after infection before G418 selection was applied.

[g] The type of proviral structure in the clone refers to the types illustrated in Fig. 7. Abbreviations: C, complete genome; A, altered genome. In general, only clones that contained one chromosome integration site could be assigned to one or another class. In the case of S102, sufficient information was available to catalog both transductants. The difference between C1 and C2 is that C1 clones contain no additional bands (i.e., cellular-AAV junction bands) when cleaved with enzymes that cut within the terminal repeats.

[h] Contained tandem integrated copies of a deleted genome.

[i] Contained tandem integrated copies of a sequence greater than full length.

[j] Contained at least one full-length integrated copy of wt AAV in addition to *dl*3-94/neo.

[k] Contained a deleted genome capable of rescue.

[l] ND, Not done.

[m] Contained 5 to 30 copies of full-length extrachromosomal *dl*52-91/neo in addition to the integrated copies.

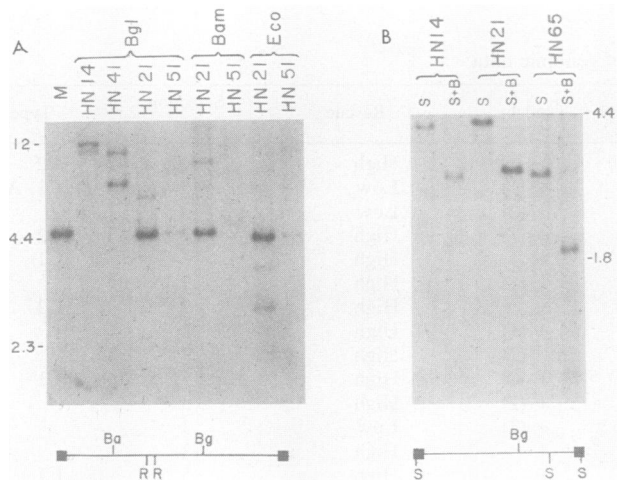

FIG. 3. Analysis of genomic DNA isolated from *dl*52-91/neo transduced D6 cell lines (H series). (A) Genomic DNA from each clone (20 μg) was digested with the indicated enzyme, electrophoresed on a 1.0% agarose gel, blotted to nitrocellulose, and probed with ^{32}P-labeled *dl*52-91/neo plasmid DNA. M is a size marker; other marker positions are indicated at the side of the gel in kilobase pairs but are not shown. A diagram of the *dl*52-91/neo viral genome is shown below the gel, indicating the positions of the restriction sites used: *Bgl*II (Bg), *Bam*HI (Ba), and *Eco*RI (R). (B) Same as panel A, except that S indicates that the DNA was digested with *Sma*I and S+B indicates a double digest with *Sma*I and *Bgl*II.

not also contain at least a partial duplication of the provirus. One of these clones (S114; Table 2), as well as one of the concatemeric clones (C2D1 and S107; Table 2 and Fig. 2), rescued a genome that contained a deletion.

Occurrence of integration at only one location at a random site within host chromatin. By using restriction enzymes that did not cut within the proviral sequences or enzymes that cut only once, we could estimate the number of discrete sites in host DNA at which integration had occurred in each clone. Most clones (74%) contained only one cellular integration site (Table 3), and this was true regardless of whether the transductant was Res$^+$ or Res$^-$ (Table 2). Only one clone

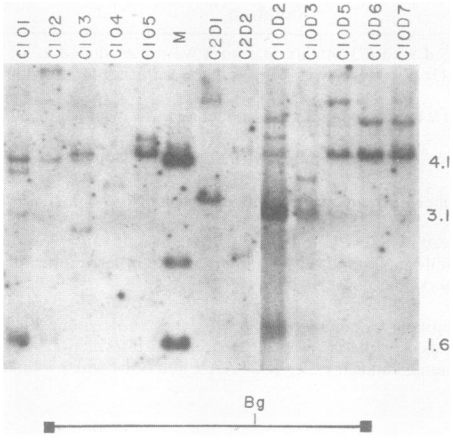

FIG. 4. Analysis of genomic DNA isolated from *dl*52-91/neo transduced D6 cell lines (C series). Genomic DNA (20 μg) from the indicated clone was digested with *Bgl*II (Bg) and analyzed as described for Fig. 3. M is a marker lane; other relevant size positions are indicated at the right of the gel.

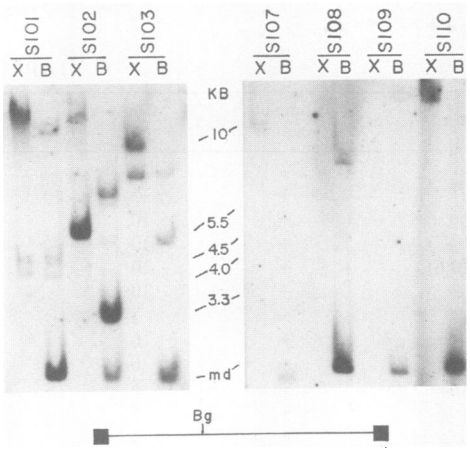

FIG. 5. Analysis of genomic DNA isolated from *dl*3-94/neo D6 transduced clones. Genomic DNA (40 μg) from the indicated clones was digested with *Xba*I (X) or *Bgl*II (B) and blotted as described for Fig. 3. The blot was probed with ^{32}P-labeled pBRNeo DNA. The positions of marker DNA fragments, as well as monomer duplex (md) *dl*3-94/neo (bp 2203), are indicated. *dl*3-94 does not have an *Xba*I site.

(Fig. 5, clone S101) contained more than two integration sites. In addition, digestion of each clone produced a unique pattern of fragments, suggesting that AAV was integrating randomly with respect to cellular sequences.

Episomal copies of the AAV genome in some clones. If a clone contained free episomal copies of an AAV recombinant genome, a characteristic set of bands should appear when genomic DNA is digested with a particular enzyme. For *dl*52-91/neo digested with *Bgl*II, these fragments would be 3.1 and 1.6 kb in length. Two clones infected with *dl*52-91/neo produced bands of these sizes (Fig. 4, clones C10D2 and C10D3). When we probed undigested DNA from these two clones, we confirmed that they did indeed contain unintegrated linear copies of *dl*52-91/neo (data not shown). We note that Cheung et al. (11) also found that a cell line containing an integrated provirus could, upon continued passage of the cell line, generate linear episomal copies of the provirus. This implies that rescue can occur in the absence of a helper virus infection.

Presence of one or two copies of the terminal palindrome in AAV-AAV junctions. The number of copies per tandem repeat was 2 to 20. In general, high-rescue clones contained longer tandem arrays than low-rescue clones did, but the correlation was not absolute (Table 2). For example, S101 contained a tandem array with approximately 10 copies but rescued poorly, whereas S109 had a three-copy tandem repeat which rescued quite well (Fig. 2 and 5; Table 2). To determine the number of copies of the AAV terminal repeat at an AAV-AAV junction within a tandem array, we digested nine clones of the S series with *Pst*I (Fig. 6). If only one copy

TABLE 3. Proviral structures

Provirus	No. with single integration/ total no. (%)	No. with concatemers/ no. Res$^+$ (%)
*dl*52-91/neo	17/23 (74%)	12/12 (100%)
*dl*3-94/neo	14/19 (74%)	12/16 (75%)
Total	31/42 (74%)	24/28 (86%)

of the AAV terminal palindrome is present at an AAV-AAV junction, *Pst*I would generate a fragment of 1.3 kb; two copies would produce a fragment of 1.4 kb. Of the nine clones examined, three contained two copies of the terminal palindrome and the remainder contained one (Table 2). There was no correlation between the type of AAV-AAV junction present and the frequency of rescue.

Location of most AAV-cellular junctions at or near the AAV terminal sequences. Previous studies of wt proviruses (11, 26) had suggested that proviral junctions with cellular DNA occurred preferentially near the termini of AAV. To determine whether this was the case with recombinant proviruses, we digested a number of clones with enzymes (*Sma*I, *Bal*I, and *Bgl*I) that cut within or near the terminal repeats of AAV (30 to 650 bp from the ends). If the digests produced only the expected internal AAV bands, then the cellular-AAV junctions must be near the ends of AAV; if additional junction bands were seen, then one of the sites near an end must be missing and recombination with cellular sequences must have occurred at an internal position within proviral sequences. We note that interpretation of these experiments was difficult for several reasons. First, the apparent absence of a site could have been due to methylation of the restriction site. Second, the absence of a clear junction band could be due to our inability to resolve it in the size range chosen for agarose gel electrophoresis. Third, the presence of a junction band might be due to a cryptic genome integrated at a different location. Despite these difficulties, the results suggested a clear trend.

Of 14 Res$^+$ proviruses examined, 11 appeared to have both junctions with cellular DNA near an AAV terminus. These were arbitrarily classified into two types, C1 and C3, and are schematically illustrated in Fig. 7. The C1 class (9 of 14) consisted of a tandem concatemer of the recombinant genome in which all of the units of the tandem array were intact and cellular DNA junctions occurred near an AAV end. The C3 class consisted of clones in which apparently only a single copy of the AAV genome had been integrated. The members of each class are listed in Table 2.

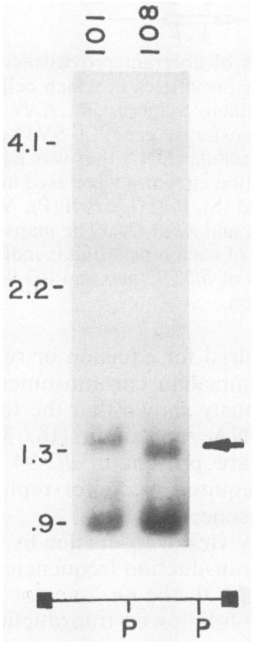

FIG. 6. Analysis of AAV-AAV junctions. DNA from the indicated S clones (40 μg) was digested with *Pst*I (P) and blotted as described for Fig. 3, except that electrophoresis was on a 1.4% agarose gel. The arrow indicates the location of the *Pst*I junction fragments.

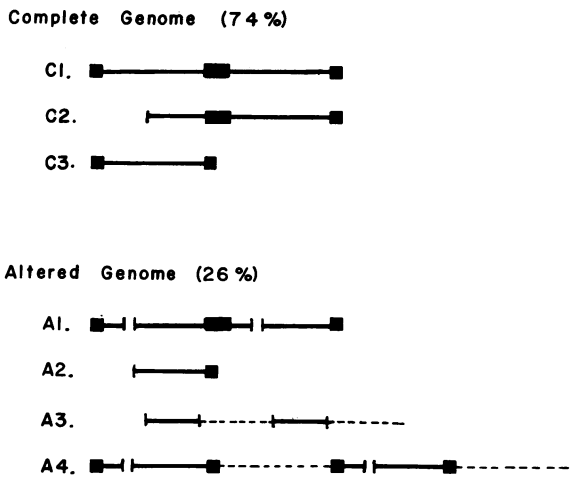

FIG. 7. Types of proviral structures. The diagram summarizes the types of proviral structures found in this study. Proviruses have been classified on the basis of structure into two major groups: C (complete genome) and A (altered genome). Group C proviruses represented 73% of the total and contained at least one complete viral genome which was Res$^+$. Group A proviruses contained altered genomes that were Res$^-$. These two major groups have been further subdivided on the basis of structure as shown the figure. For example, C1 proviruses contained a concatemer in which both cellular-AAV junctions were at an AAV end, C2 proviruses contained one cellular junction at an AAV end and the other at a random position with respect to AAV sequences, whereas C3 proviruses contained only a single copy of the AAV recombinant genome. Symbols: ——, AAV sequences; – – – – –, flanking cellular DNA which has been tandemly repeated; ■, AAV terminal repeats; |, sites of deletion-recombination. Examples of each structure are indicated in Table 2 under the designation Type; some of the altered structures are shown in more detail in Fig. 8. The fact that AAV-AAV junctions frequently contained only one copy of the terminal palindrome is not shown in this diagram.

The three remaining Res$^+$ clones appeared to have proviruses that had recombined with cellular DNA at internal sequences. In these three clones, only one of the cellular DNA junctions occurred at an internal AAV position; the other junction was near an AAV end. We arbitrarily called this type of structure C2 (Fig. 7; Table 2). For example, HN51 was approximately 1.6 genomes in length and consisted of a tandem repeat in which one end of the concatemer was joined to cellular DNA near an AAV end and the other had recombined between the *Bam*HI and *Eco*RI sites of *dl*52-91/neo (Fig. 3A).

Presence of deleted proviruses and one of several different proviral structures in Res$^-$ clones. Virtually all the Res$^-$ clones harbored a provirus that contained a deletion (Table 2). Some of these clones (5 of 12) contained less than one copy of the input viral genome and were found to have deletions that removed at least one end of the genome (Fig. 7, provirus A2; Table 2). The remaining Res$^-$ clones had more complex proviral structures. One clone, C102 (Fig. 4; Table 2), was found to be a tandem repeat of a *dl*52-91/neo genome that had suffered an internal deletion within the AAV *rep* gene (Fig. 7, provirus A1). This provirus was similar to the Res$^+$ clone S107 (Fig. 2 and 5; Table 2) and C2D1 (Fig. 4; Table 2), which also contained a tandem repeat

of an internal deletion. Finally, several of the Res$^-$ clones contained tandem repeats of defective proviruses in which both the proviral DNA and flanking cellular DNA had been reiterated (Fig. 7, proviruses A3 and A4). For example, when HN14 was digested with *Sma*I, an enzyme that cuts within the AAV terminal repeat, the clone was found to have approximately six copies of a *dl*52-91/neo genome that contained a small deletion in the *rep* gene (Fig. 3B). This was confirmed by digestion with *Bal*I and *Pst*I (results not shown). However, digestion of HN14 with single-cut enzymes such as *Bgl*II (Fig. 3A) or *Bam*HI (results not shown) produced fragments much larger than the one expected from a simple tandem repeat of *dl*52-91/neo. We concluded that HN14 consisted of a tandem repeat of proviral DNA and flanking cellular DNA (Fig. 7, provirus A4). The repeat unit appeared to be 22 kb. A similar analysis was done for HN41 (Fig. 3A), HN65 (Fig. 3B), and one of two tandem arrays present in S102 (Fig. 5). In S102 the aberrant tandem array could be distinguished from the conventional tandem repeat because of its high copy number. The simplest structures for all four of these cases are diagramed in Fig. 8. In each case we have indicated the enzymes that were used to derive the structure. Although each structure is consistent with the restriction fragment pattern that we saw, it represents only one of several possible structures that could have been proposed.

Possibility of more than one provirus type in a cell line. Two cell lines contained both a recombinant AAV/neo provirus and a wt AAV genome. For example, S101 (Fig. 2) contained a *dl*3-94/neo provirus that was rescued when the cells were superinfected with only adenovirus. This was subsequently shown to be due to the presence of a wt AAV provirus that was also capable of rescue and could complement the *rep* defect of *dl*3-94/neo. The presence of a wt provirus in S101 and S118 (Table 2) was presumably due to the fact that the *dl*3-94/neo viral stock used in this study was contaminated with wt virus. We presume that some of the clones that contained the *rep*$^+$ *dl*52-91/neo provirus also contained wt AAV proviruses, but because the size of *dl*52-91/neo is virtually identical to that of wt AAV and because *dl*52-91/neo is capable of replicating autonomously following adenovirus rescue, no attempt was made to identify these clones.

This observation raised the question of whether an endogenous AAV provirus would inhibit the integration of superinfecting AAV genomes. To find out, we infected a D5 cell line (which contains a wt AAV provirus) with *dl*52-91/neo. The wt proviral line (D5) had been previously characterized as a low-rescue line (18) which contained two to five tandem copies of wt DNA (11). We found no difference in the transduction or rescue frequencies between the parental D6 cell line, which contained no AAV DNA, and the D5 line (data not shown). We concluded that there was no superinfection inhibition for the integration of AAV proviruses.

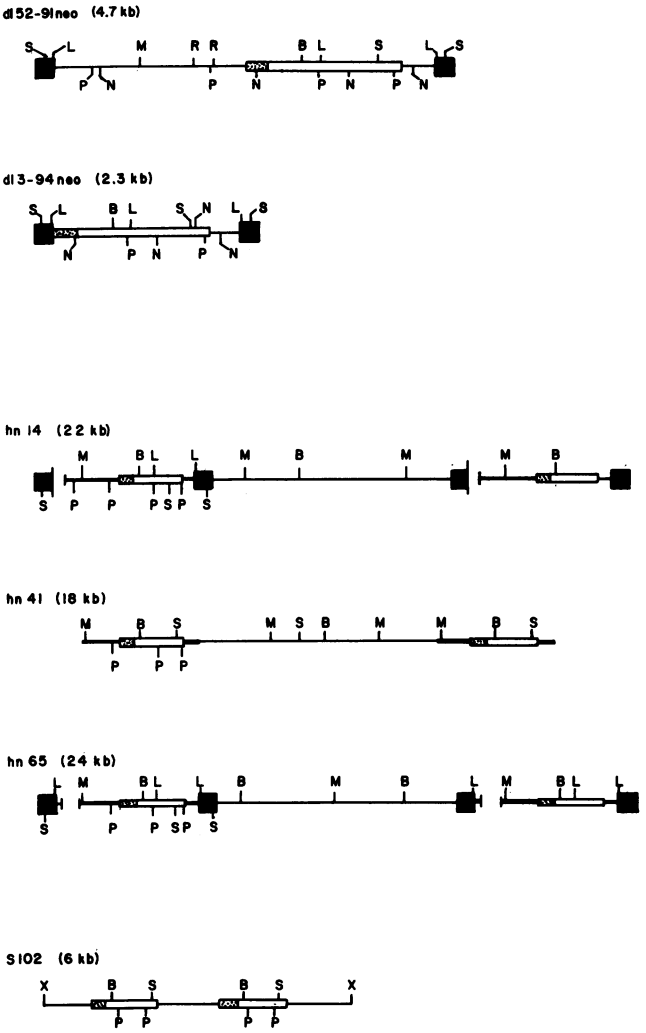

FIG. 8. Structures of aberrant proviruses. Probable restriction enzyme maps of four proviruses in which cellular as well as AAV DNA had been amplified. Symbols: ■, AAV terminal repeats; □, neomycin phosphotransferase gene; □, SV40 enhancer; ▬, AAV sequences; _____, cellular DNA that was part of the repeat unit. The following restriction enzymes were used in the determination of these structures: *Sma*I (S), *Bal*I (L), *Pst*I (P), *Nco*I (N), *Bam*HI (M), *Eco*RI (R), *Bgl*II (B), and *Xba*I (X). The maps are not to scale, and the approximate size of each repeat unit is indicated in parentheses. The restriction maps of *dl*52-92/neo and *dl*3-94/neo are included at the top for comparison.

DISCUSSION

Presence of all essential *cis*-active sequences at the ends of *dl*3-94. *dl*3-94 contains only the AAV terminal repeats and the 139 bp of nonrepeated sequence adjacent to the right-hand terminal repeat. The fact that foreign DNA cloned into *dl*3-94 can be successfully packaged into virions and integrated into host chromosomes indicates that the AAV packaging signal (*pac*) and a possible integration signal (*int*) are contained in the 284 bp of AAV sequences retained in *dl*3-94. Furthermore, the fact that most of the *dl*3-94/neo transductants could be rescued implies that the terminal repeats also contain a site required for excision or rescue (*res*) of AAV genomes from mammalian chromosomes. Finally, we and others have previously shown that the terminal repeats are the origins for DNA replication (16, 37, 39). Thus, the sequences which are present in *dl*3-94 contain all of the essential signals required in *cis* for replication, packaging, integration, and rescue.

Inhibition of AAV viral transduction by the *rep* gene. When we compared the transduction frequencies of *dl*3-94/neo and *dl*52-91/neo at low MOI, the *rep*$^+$ vector *dl*52-91/neo had an approximately 200-fold-lower transduction frequency. We conclude that it is very unlikely that *rep* gene expression is required for integration of the viral genome; indeed, the presence of the *rep* gene seems to inhibit transduction.

We are not certain how *rep* inhibits transduction. One possible explanation is that the *rep* gene product(s) is syn-

thesized at some level following AAV infection in the absence of helper virus and that it subsequently either inhibits the expression of the SV40 promoter or inhibits integration itself. This would confirm the recent observations of Labow et al. (24, 25) and Tratschin et al. (46) that the AAV Rep protein can repress gene expression under some conditions. In particular, Labow et al. (24) demonstrated that the wt AAV *rep* gene, but not mutant Rep proteins, had an inhibitory effect on calcium phosphate-mediated transformation when a selectable marker driven by an SV40, herpesvirus *tk*, or mouse metallothionein promoter was used.

The repression by *rep* of heterologous promoters raised the question of whether AAV viral infection at high MOI was lethal to cells. Laughlin et al. (26) reported no difference in plating efficiency of cells infected by AAV at an MOI of 100. We infected nontransformed cells with an MOI of 1,000 and also saw no effect on cell viability (data not shown). Thus, repression by *rep* either is a transient phenomenon from which the cell recovers or affects only some cellular promoters. The fact that there was no difference in the transduction frequency of a cell line carrying a wt AAV provirus (D5) and that of its parental line (D6) suggests that *rep*-mediated repression may be transient and that once the provirus is integrated, *rep* expression is reduced.

Presence of intact provirus in most transduced lines. A major question about AAV integration has been whether there is a specific mechanism of integration with respect to AAV sequences. Previous studies of wt AAV integration have relied primarily on the ability to rescue an intact genome to select for cells that contained a provirus (3, 11, 26). Because of the nature of the selection, only cells that contained an intact provirus were examined. In this study we used the neomycin resistance gene as the selectable marker, and the AAV sequences were nonessential. If there were no mechanism for maintaining the integrity of AAV proviral sequences, we would have expected the frequency of rescue to be low. In fact, the frequency of rescue in most experiments was greater than 80%. Furthermore, when we compared *dl*52-91/neo, and *dl*3-94/neo; there was no significant difference in their ability to integrate an intact provirus, even though more than half of *dl*52-91/neo was nonessential during G418 selection. We conclude, then, that there is a mechanism for maintaining the integrity of AAV DNA during integration which involves the AAV terminal repeats.

Integration of intact proviruses by AAV via concatemers. Most of the clones (86%) that had an intact viral genome (Res^+) contained a proviral concatemer. When we examined Res^- clones, most of these also contained concatemers (although with defective genomes) and the remainder were single-copy clones. Concatemers therefore appear to be an essential feature of AAV integration, and any model for AAV integration must account for their formation. We note that Cheung et al. (11) and Laughlin et al. (26) saw similar proviral structures with wt AAV. However, in a study by Tratschin et al. (45), four of four proviruses in the adenovirus-transformed 293 cell line were not concatemers. In 293 cells the constitutive expression of the adenovirus transforming genes may have intefered with normal AAV integration (27). Recent studies (47; S. K. McLaughlin and N. Muzyczka, unpublished results) suggest that 293 cells can support limited AAV DNA replication and therefore that 293 cells may provide an environment that is permissive for lytic infection.

One reason why concatemers may be necessary is that the recombination between AAV sequences and cellular DNA may not be precise. Although most cellular-AAV junctions appeared to be near an AAV end, in some cases integration had clearly occurred randomly with respect to AAV sequences. Furthermore, preliminary evidence suggests that at the DNA sequence level, a substantial amount of the terminal AAV repeats at the ends of a concatemer may be deleted during integration (McLaughlin and Muzyczka, unpublished results). The formation of a concatemer, then, would be a mechanism for maintaining the integrity of at least one copy of the AAV genome during integration.

DNA replication rather than recombination as cause of AAV concatemers. AAV proviral concatemers are probably the result of the amplification of a single input genome rather than the joining of several input molecules. We reached this conclusion because of the following observations. (i) The formation of concatemers occurred at approximately the same frequency, regardless of whether transduction occurred at low or high MOI (0.03 to 10 infectious units). (ii) Although we had a wt virion contaminant in both of our stocks and occasionally found cell lines which contained both wt and recombinant proviruses, we never found a proviral array which contained a mixture of the two genomes at the same integration site. (iii) We never found a concatemer which contained inverted repeats (head to head or tail to tail). All of the concatemers that were found consisted of tandem genomic arrays. (iv) At least two defective proviral concatemers were found that consisted of tandem arrays of a genome with an internal deletion. It is unlikely that 2 to 10 copies of the same deleted genome could fortuitously infect the same cell. (v) When we examined nine cell lines for the number of terminal repeats at each AAV-AAV junction, there were either one or two copies of the terminal 125-bp repeat. We never found a mixture of one and two copies as might be expected if the concatemers had formed by recombination. Finally, although all of these observations suggest that AAV proviral concatemers occur by a replicative amplification, it should be noted that recombination followed by gene conversion could also account for many of our observations.

AAV replication by a different mechanism during integration. During lytic infections AAV replicates by a mechanism that leads to the synthesis of head-to-head or tail-to-tail concatemers. Therefore, the mechanism of replication used during integration to synthesize proviral concatemers must be different from that used during lytic infections. This point was also made by Laughlin et al. (26). The features that must be explained by any mechanism of AAV integration (Fig. 8) are (i) the conversion of single-stranded input DNA to a duplex form, (ii) the formation of tandem genomic repeats, (iii) the presence of one or two copies of the 125-bp terminal palindrome at AAV-AAV junctions, (iv) the presence of AAV termini at most junctions with cellular DNA, (v) the fact that some proviruses can be rescued more efficiently than others, and (vi) the fact that the AAV termini contain the sequences required in *cis* for integration. A variety of models can be constructed to account for these observations, and they fall into two general classes: (i) AAV DNA circularizes and undergoes rolling-circle replication by using the terminal repeat as an origin, and (ii) a monomer-length input genome integrates (possibly as a single-stranded DNA molecule) and then is precisely amplified in place. With respect to the second model, it is worth mentioning that some of the *res* clones contained flanking cellular sequences as part of the concatemer and that Cheung et al. (11) also found cellular DNA within a wt concatemer. Interestingly, both types of models assume that the AAV termini are

recognized by cellular enzymes as origins for DNA replication.

The significance of high and low rescue. All intact proviruses could be rescued, but the efficiency of rescue was quite variable and did not correlate with any obvious structural feature. It is unlikely that cellular-AAV junctions have a major effect on rescue because, presumably, the local environment of the internal AAV copies is similar in all concatemers. The phenomenon of low rescue is reminiscent of the low rescue efficiency observed with recombinant plasmids that contain terminal deletions (29, 37). The replication of the deletion mutants is delayed following transfection either because they lack an AAV sequence that facilitates excision (Gottlieb and Muzyczka, submitted) or because they have to reconstruct a wt origin by gene conversion (37). It is possible, then, that similar deletions occur within the terminal palindromes at AAV-AAV junctions of proviral concatemers. Such deletions could account for the fact that some clones rescue poorly.

***dl*3-94 as a vector.** We and others have commented on the potential of AAV vectors (18, 26, 45). The *dl*3-94 family of vectors should be useful for three reasons. First, they allow maximum room for insertion of foreign DNA sequences. Second, the presence of two cloning sites separated by a polyadenylation signal should allow the insertion of a selectable marker and a second gene under separate control. Third, removal of the *rep* gene apparently increases the transduction frequency.

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