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Latent Infection of KB Cells with Adeno-Associated Virus Type 2

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Adeno-associated virus (AAV) is a prevalent human virus whose replication requires factors provided by a coinfecting helper virus. AAV can establish latent infections in vitro by integration of the AAV genome into cellular DNA. To study the process of integration as well as the rescue of AAV replication in latently infected cells after superinfection with a helper virus, we established a panel of independently derived latently infected cell clones. KB cells were infected with a high multiplicity of AAV in the absence of helper virus, cloned, and passaged to dilute out input AAV genomes. AAV DNA replication and protein synthesis were rescued from more than 10% of the KB cell clones after superinfection with adenovirus type 5 (Ad5) or herpes simplex virus types 1 or 2. In the absence of helper virus, there was no detectable expression of AAV-specific RNA or proteins in the latently infected cell clones. Ad5 superinfection also resulted in the production of infectious AAV in most cases. All mutant adenoviruses tested that were able to help AAV DNA replication in a coinfection were also able to rescue AAV from the latently infected cells, although one mutant, Ad5*hr*6, was less efficient at AAV rescue. Analysis of high-molecular-weight cellular DNA indicated that AAV sequences were integrated into the cell genome. The restriction enzyme digestion patterns of the cellular DNA were consistent with colinear integration of the AAV genome, with the viral termini present at the cell-virus junction. In addition, many of the cell lines appeared to contain head-to-tail concatemers of the AAV genome. The understanding of the integration of AAV DNA is increasingly important since AAV-based vectors have many advantages for gene transduction in vitro and in vivo.

Adeno-associated virus (AAV) is a ubiquitous defective human parvovirus which can replicate only in cells coinfected with a helper adenovirus or herpesvirus (2, 9, 10, 47). Although almost two-thirds of the adult population of the United States has serological evidence of exposure (8), there is no evidence of any pathology associated with AAV infection. In fact, AAV may interfere with some aspects of the pathology of its helper viruses in vivo as it has been demonstrated to do in vitro (12, 16, 28, 36, 42, 43). AAV infection in the absence of helper virus can result in the establishment of a latent infection both in vitro (4, 14, 21, 29) and in vivo (16, 29). The latent state is maintained in cell culture by integration of the viral DNA into cellular DNA (14). Superinfection of latently infected cells with a helper adenovirus results in the rescue and replication of the AAV genome. Because of its apparently efficient integration, nonpathogenicity, and ability to be rescued, AAV has recently been adapted for use as a eucaryotic integrative vector (24, 55; C. A. Laughlin, manuscript in preparation). An AAV-based vector shares many advantages with retrovirus vectors (13, 52, 53) for both clinical and research applications, but the nonpathogenicity of AAV may make it the best choice for clinical gene transduction. Therefore, it is essential that the process of latent infection with AAV be understood.

The first suggestion that AAV could establish latent infections came from the discovery of AAV contamination of some adenovirus type 12 (Ad12) stocks grown in primary human and African green monkey embryonic kidney cells (27). This report was soon followed by the in vitro establishment of latent infection of Detroit 6 cells with AAV1, AAV2, and AAV3 (29). Detroit 6 cells were infected with high multiplicities of AAV in the absence of helper virus coinfection. Superinfection of these cultures with adenovirus 47 passages after initial infection with AAV resulted in the rescue of AAV protein synthesis as measured by a fluorescent-focus assay. AAV DNA was demonstrated to be associated with cellular DNA in Detroit 6 cells latently infected with AAV2 by a solution hybridization assay (5) and in KB cells latently infected with AAV1 by a DNA network analysis (21). In a more detailed report, the integration pattern of AAV2 sequences was determined for one of the Detroit 6 clones (14). Three to five copies of integrated viral sequences were present in one or a few sites as head-to-tail tandem repeats. Viral terminal sequences were joined to the opposite viral terminus or present in higher-molecular-weight bands, presumably as a consequence of covalently attached cellular sequences. Therefore, in this cell line the viral sequences appear to have recombined with cellular DNA via the viral termini. Since it has been previously shown that in the absence of helper virus AAV penetrates the nucleus and is uncoated but is not transcribed (48), the integration of the viral genome is probably accomplished by cellular mechanisms.

These findings suggest that the integration of concatemeric sequences which are colinear with the viral genome and the presence of the viral genomic termini at the cell-virus junctions are general features of AAV DNA integration in latent infection. It is important to extend these data since (i) AAV latent infection apparently occurs in vivo and AAV is a ubiquitous human virus, (ii) integration apparently involves normal cellular recombination systems, and (iii) an understanding of latent AAV infection will result in more effective use of AAV-based eucaryotic vectors. Therefore, we determined the efficiency of establishment of latent infection of AAV2 in KB cells and characterized several of the resultant clones.

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MATERIALS AND METHODS

Cells and viruses. KB-3 monolayer and 293 cells, an adenovirus-transformed human cell line (20), were maintained in Eagle minimal essential medium supplemented with 10% fetal bovine serum. The cultures were determined to be free of mycoplasma by the hybridization assay in the Gen-Probe mycoplasma detection kit. Stocks of wild-type Ad5 and AAV2 were grown in KB spinner cells as previously described (33). AAV2 stocks were purified by equilibrium banding in CsCl (33), and infectivity was determined by a fluorescent-focus assay (11) by using antiserum provided by B. J. Carter and F. Jay. Stocks of the Ad5 host-range mutants *hr*6 and *hr*13 (provided by J. Williams), and *sub*316 (provided by T. Shenk) were grown in 293 cells. Stocks of the temperature-sensitive mutants Ad5*ts*215 (provided by W. Doerfler) and Ad5*ts*149 (provided by H. Ginsberg) were prepared in KB cells as described previously (39). Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) were provided by I. Hay, and new stocks were grown on Vero cells. All viruses were adsorbed to cells in a small volume as previously described (11).

Single-cell cloning was accomplished by distributing well-trypsinized cells in 96-well microtiter dishes at a cell concentration such that only approximately one-fourth of the wells received a cell. The wells were microscopically checked to ascertain that only one colony arose in each well. The cells were plated in conditioned medium which was prepared by removing the medium from subconfluent KB cells, filtering it, and supplementing it with 10% fetal bovine serum. Individual clones were expanded and stored in liquid nitrogen.

Assay of viral DNA and infectivity. AAV DNA was isolated from infected cells by using a previously described modification of the Hirt procedure (11, 25). Hirt supernatants were electrophoresed on 0.8% agarose gels, blotted onto nitrocellulose or nylon membranes, and hybridized with a nick-translated AAV-specific probe by standard techniques (37). Because of a shortage of AAV-specific antiserum, the fluorescent-focus assay was used only to determine AAV stock titers. To determine the infectivity of AAV rescued from latently infected cells, helper-virus-infected cells were harvested at 48 h postinfection (p.i.). The infected cells were either frozen in their medium or pelleted and suspended in 1 ml of phosphate-buffered saline (with cations) per 60-mm dish. The sample was frozen and thawed three times and heated to 60°C for 1 h to inactivate the helper virus. A sample was used, along with a helper virus, to infect subconfluent KB cells, Hirt supernatants were prepared as described above, and the resulting blots were probed either with AAV DNA extracted from CsCl-banded virions and further purified on a sucrose gradient or with a gel-purified restriction fragment of the AAV-containing plasmid pAV2 (34).

Preparation of RNA from infected cells. Infected cell RNA was prepared by a modification of the guanidinium isothiocyanate method (15). RNA was prepared from 15 150-cm confluent monolayer cultures. The cells were harvested by trypsinization and suspended at a concentration of 10^8 cells per 75 ml of phosphate-buffered saline. A solution was made which contained 4 M guanidinium isothiocyanate, 50 mM sodium citrate, 10 mM EDTA, 2% Sarkosyl (CIBA-GEIGY Corp.), and 133 mM β-mercaptoethanol. All water and solutions used were treated with diethyl pyrocarbonate to inactive RNase. To each 10^7 cells, 1 ml of the prepared guanidinium isothiocyanate solution was added, and the suspension was vortexed. Cesium chloride was added at a concentration of 1 g/2.5 ml of cell homogenate, and the solution was distributed into polyallomer SW28 tubes over a cushion of 7 ml of 5.7 M CsCl in 0.1 M EDTA (pH 7.5). Centrifugation was at 80,000 × *g* for 48 h at 20°C in an SW28 rotor. The supernatant was discarded, and the RNA pellet was dissolved in 1 ml of sterile 10 mM Tris (pH 7.4)–5 mM EDTA–1% sodium dodecyl sulfate.

The RNA was extracted twice with a mixture of four parts chloroform to one part *l*-butanol. To the combined aqueous phases were added 0.1 volume of 3 M sodium acetate and 2.2 volumes of ethanol, and the tubes were incubated at −20°C for 2 h. The RNA was pelleted, the pellets were dissolved in 1 ml of diethyl pyrocarbonate-treated water, and the ethanol precipitation was repeated. The RNA was stored in 70% ethanol at −70°C.

Poly(A)$^+$ RNA was isolated by batch adsorption and elution from oligo(dT) cellulose (37). Samples of poly(A)$^+$ RNA (20 μg per track) were electrophoresed on formamide agarose gels as previously described (35), blotted onto nitrocellulose, and hybridized by standard techniques (37). Restriction-digested plasmid DNA (2 μg per track) was used as molecular-weight markers.

Analysis of cellular DNA. Cellular DNA was isolated from individual cell clones for restriction enzyme analysis. Cells were trypsinized, pelleted, and suspended in 0.01 M Tris (pH 8.0) at a density of 10^6 cells per ml, and frozen. After being thawed, the cells were incubated for 2 h at 37°C with 0.5% sodium dodecyl sulfate–20 μg of proteinase K (Sigma Chemical Co.) per ml. Nucleic acids were isolated by sequential extractions with phenol, phenol-chloroform (1:1), and chloroform followed by ethanol precipitation. Nucleic acids were pelleted, suspended in 1 mM EDTA–0.01 M Tris (pH 8.0), and incubated with 50 μg of RNase A per ml, followed by a second proteinase K digestion, two further phenol–chloroform extractions, and precipitation in ethanol. Pelleted DNA was resuspended, and its concentration was determined spectrophotometrically. For analysis of the AAV integration pattern, cellular DNAs (20 μg) were digested overnight with an excess of the appropriate restriction enzyme. To allow assessment of the completeness of digestion and to provide markers for viral fragments, AAV DNA was added to 20 μg of calf thymus DNA and similarly digested. Samples were electrophoresed on 0.7% agarose gels in Tris acetate buffer (37) for 60 h at 30 mA constant current. The buffer was changed several times during the run. The gels were blotted onto nylon membranes and hybridized as described above.

RESULTS

Establishment of latent infection. The efficiency of establishment of latent infection of KB cells by AAV2 was determined in two different experiments. Initially, KB cells were infected with AAV2 at a multiplicity of infection of 200 fluorescent focus-forming units per cell, and the cells were passaged 10 times at a 1:10 split ratio to ensure that all input viral genomes were diluted out. At this stage, the cells were cloned by limiting dilution in 96-well microtiter dishes. Individual cell clones were expanded, and a portion was tested for the appearance of AAV capsid antigens after adenovirus superinfection by an indirect immunofluorescence assay. Clones were considered latently infected if at least 10% of the cells stained positively for AAV2 capsid antigens after infection with adenovirus helper virus. Therefore, any cell clone would be counted as negative which had integrated AAV DNA that was neither rescued after adeno-

TABLE 1. Rescue of AAV from cloned AAV-infected KB cells

Expt no.	No. of clones tested	No. rescued	% Rescued
1[a]	41	3	7.3
2[b]	63	9	14.3

[a] In experiment 1, a clone was considered positive for rescue if at least 10% of the cells were positive in an indirect immunofluorescence assay for AAV capsid antigens after adenovirus infection.

[b] In experiment 2, a clone was considered positive for rescue if Hirt supernatants prepared from adenovirus-infected cells contained AAV DNA.

virus infection nor efficiently transcribed in the integrated state. Of the 41 cell clones tested that gave unambiguous results, 3 (7.3%) were latently infected with AAV (Table 1).

The main criticism of this method of determining efficiency of establishment of latent infection is that an integrated AAV genome might affect the viability of the infected cell. This problem is also inherent in the previously reported efficiency determination of 30% of establishment of latent AAV infection in Detroit 6 cells (14). Therefore, the experiment was repeated with the modification of cloning the infected cells immediately after the 2-h adsorption period. There was no difference in the cloning efficiencies of the AAV-infected KB cells and mock-infected controls, which suggests that AAV infection does not have a significant effect on cell viability. When a clone derived from the AAV-infected population had reached a diameter of about 5 mm, it was picked and passaged, as before, 10 times at a 1:10 split ratio. KB cell clones were considered latently infected if Hirt supernatants prepared from the clones after adenovirus infection contained AAV DNA as detected by electrophoresis, Southern blotting, and hybridization as described in Materials and Methods. Again, a cell clone which had integrated viral sequences which could not be rescued would be considered negative. Of the 63 clones tested 9 (14.3%) were latently infected (Table 1). Therefore, it can be concluded that a high multiplicity of infection of KB cells with AAV2 results in latent infection of more than 10% of the cells.

Rescue of AAV DNA and infectious virus from latently infected cells. It seemed likely that AAV sequences could be rescued by adenovirus in all of the latently infected cell clones since the synthesis of AAV antigens or replication of viral DNA after adenovirus superinfection was the basis for initial identification of the clones. However, the capsid antigen observed could have been transcribed from integrated unrescuable genomes. Therefore, rescue of AAV DNA replication was analyzed directly by the demonstration of AAV replicative forms in Hirt extracts of Ad-infected latently infected cell clones. A representative autoradiogram demonstrating rescued viral sequences is shown in Fig. 1. In panel A, it is clear that both AAV duplex monomer and single-stranded monomer DNA (positions marked by arrows) have been rescued by adenovirus superinfection in clones M21, M32, M34, and M69. AAV was not rescued from M50 and M77, two cell lines which did not contain integrated viral sequences (data not shown). AAV DNA was rescued in M19, but the duplex monomer replicative form showed an increased electrophoretic mobility, and the single-stranded DNA showed a decreased mobility. Further restriction enzyme and electrophoretic analysis demonstrated that the AAV genome in M19 cells has an internal deletion of about 300 nucleotides. Panel B demonstrates that HSV-1 (lane 3) could also rescue latent AAV DNA genome replication and, in fact, was more efficient than was Ad5 (lane 2).

One report suggested that an adenovirus mutant which was able to help AAV DNA replication efficiently was not able to rescue AAV from a latently infected Detroit 6 cell clone (40). Therefore, the ability of several adenovirus mutants and HSV to rescue AAV DNA replication in several of the latently infected clones was compared by analysis of AAV DNA in Hirt supernatants. Three latently infected cell lines were tested with all helper viruses to ensure that the observed effects were general. The results from three separate experiments are summarized in Table 2. As a control, the ability of Ad5 and HSV-1 to rescue AAV DNA replication was demonstrated (Table 2). The AAV DNA replication seen in the adenovirus- and HSV-infected cultures did not result from contaminated helper virus stocks, since Ad5- and HSV-1-infected KB cells and 293 cells were negative for AAV DNA. Hirt supernatants prepared from mock-infected, latently infected cells (data not shown) and AAV2-superinfected cells (Table 2) also showed no evidence of AAV DNA replication. The first mutant tested, Ad5*ts*215, had a lesion in a late gene involved in the packaging of adenovirus virions. This mutant has been previously shown to help AAV replication (38, 39) and, as expected, it was able to rescue AAV DNA replication in the latently infected cells. Similarly Ad5*ts*149, with a mutation in the adenovirus DNA polymerase (19, 39, 51), has previously been shown to help AAV replication and also efficiently rescued AAV DNA replication at the nonpermissive temperature in all the latently infected clones tested.

Because of the report that a mutant in adenovirus region

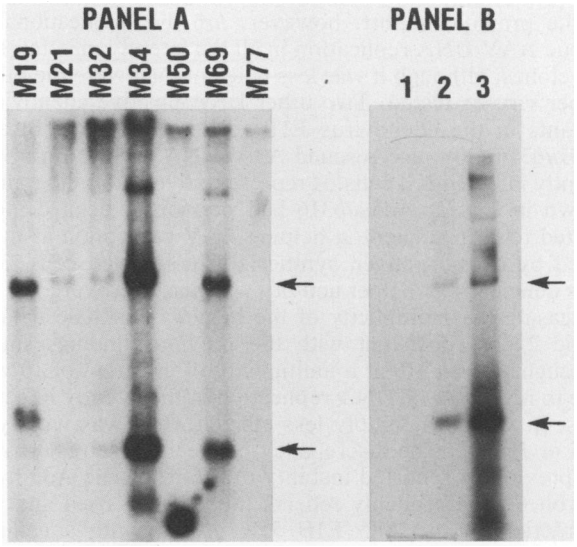

FIG. 1. KB cell clones latently infected with AAV2 were superinfected with either Ad5 or HSV-1. Viral DNA was extracted at 20 h p.i. by a modification of the Hirt procedure as described in Materials and Methods. Samples of the Hirt supernatants were electrophoresed on 0.8% agarose gels, blotted onto nylon membranes, and hybridized with nick-translated pAV2 DNA. pAV2 contains the entire AAV genome inserted into pBR322 with *Bgl*II linkers (32). The upper and lower arrows mark the position of the AAV double-stranded monomer replicative form and the single-stranded progeny genome, respectively. (A) Result of screening several clones (derived from experiment 2 of Table 1) after adenovirus superinfection. (B) Rescue of AAV DNA replication in clone C178 (derived from experiment 1 in Table 1) after mock infection (lane 1) and superinfection with Ad5 (lane 2) or HSV-1 (lane 3).

TABLE 2. Rescue of AAV DNA replication in latently infected cells by mutant and wild-type helper viruses

Virus	AAV DNA in cell line[a]				
	KB	293	Cl78	M69	M104
Ad5	−	−	+ +	+ + +	+ + +
Ad5 + AAV2	+ + +	+ + +	+ + +	+ + +	+ + +
AAV2	−	−	−	−	−
HSV-1	−	−	+ +	+ + +	+ + +
HSV-1 + AAV2	+ +	+ + +	ND[b]	ND	ND
Ad5*ts*215	ND	ND	+ + +	+ + +	ND
Ad5*ts*149	ND	ND	+ +	+ + +	ND
Ad5*hr*6	ND	ND	+	+	+
Ad5*hr*6 + AAV2	+ +	+ + +	ND	ND	ND
Ad5*hr*13	ND	ND	+	+ +	+ +
Ad5*hr*13 + AAV2	+	+ + +	ND	ND	ND
Ad5*sub*316	ND	ND	+ +	+	+
Ad5*sub*316 + AAV2	+	+ + +	ND	ND	ND

[a] 5×10^5 cells were infected with the indicated helper virus at a multiplicity of infection of 10. Hirt supernatants were isolated at 20 h p.i. and analyzed for the presence of AAV DNA as described in Materials and Methods. −, No evidence of DNA after long exposure of the autoradiograph. Positive samples range from + (AAV DNA evident after long exposure) to + + + (AAV DNA evident after very short exposure).

[b] ND, Not done.

E1B, *hr*6, was unable to rescue AAV DNA replication in latently infected Detroit 6 cells (40), the ability of this mutant to rescue AAV DNA replication in the latently infected KB cells was tested. *hr*6-infected cells do not synthesize the E1B 55,000-molecular-weight protein (55K protein), although they do express the Ad5 E1B 21K protein (22, 56). *hr*6 was able, as expected, to help AAV DNA replication in KB and 293 cells coinfected with *hr*6 and AAV (Table 2). In contrast to the previous report, however, *hr*6 did unquestionably rescue AAV DNA replication in all the latently infected KB cell clones, although it was less efficient than were the other helper viruses tested. Two other DNA-positive adenovirus mutants in the adenovirus E1B region, *hr*13 (26, 56) and Ad5*sub*316 (30), also rescued AAV DNA replication in the latently infected KB cells. A representative autoradiogram is shown in Fig. 2. Ad5*sub*316 had previously been demonstrated to be inefficient at helping AAV replication as measured by capsid antigen synthesis in coinfected cells (32). This deficiency of helper activity was partially overcome by increasing the multiplicity of the helper virus. The data in Table 2 are consistent with the previous findings since, although Ad5*sub*316 at a multiplicity of infection of 10 was able to rescue AAV DNA replication in the latently infected cells, it was considerably less efficient than was wild-type Ad5 or HSV-1. The discrepancy between these results and the previously reported inability of *hr*6 to rescue Ad5 from Detroit 6 cells probably reflects the cell line used and the interaction of the Ad5 E1B 55K protein with a cellular function. It has been clearly demonstrated that the adenovirus E1B region is not absolutely required for adenovirus replication and that E1B mutants are viable if cells are infected at high multiplicities (30). Therefore, the expression of E1B viral products increases the efficiency of infection, probably as a result of interaction with a cellular factor. Similarly, expression of the Ad5 E1B 55K protein may not be essential for the rescue of AAV DNA replication in latently infected cells but may significantly increase its efficiency in some cell lines (Detroit) that contain either an active inhibitor or low levels of an inducible activity necessary for rescue of AAV DNA replication.

Since AAV DNA replication was efficiently rescued in the latently infected KB cell clones, the production of infectious AAV virus was tested. Latently infected cells were infected with helper Ad5, and the infected cells were harvested at 72 h and processed as described in Materials and Methods. Dilutions of these harvests were used to infect KB cells in the presence of helper virus. Relative infectivity was measured by the presence of AAV DNA in Hirt supernatants. Cells coinfected with AAV2 and Ad5 were included in the initial infection as a positive control, and mock-infected cells as well as cells infected with AAV2 without helper virus were negative controls. In five of the six clones tested, rescue of AAV DNA replication by adenovirus superinfection resulted in the production of infectious virus with normal electrophoretic mobility (Table 3). No infectious AAV was rescued from cell line M19, but the AAV DNA rescued in this cell line contains a deletion (Fig. 1). Although HSV-1 was consistently as or more efficient than was adenovirus in rescuing AAV DNA replication (Fig. 1, Table 2), there was no detectable replication of infectious progeny virus in the HSV-1-superinfected, latently infected clones (data not shown). The inability of HSV to efficiently provide complete helper functions for AAV replication in productive coinfections is well documented (1, 7, 33), although one group has reported that HSV was as efficient a helper as Ad2 in KB cells (9).

Expression of AAV genes in a latently infected clone. One of the latently infected cell lines was tested for evidence of expression of the AAV genome. Indirect immunofluorescence of the three latently infected cell lines isolated in experiment 1 (Table 1) showed no evidence of virus capsid antigen synthesis in the absence of helper virus superinfection (data not shown). One cell line, C178, was also examined for the presence of virus-specific transcripts. Poly(A)$^+$ RNA was isolated from C178 cells with and without adenovirus infection and from KB cells infected with adenovirus or with adenovirus and AAV. All RNA samples were analyzed by electrophoresis in denaturing conditions, North-

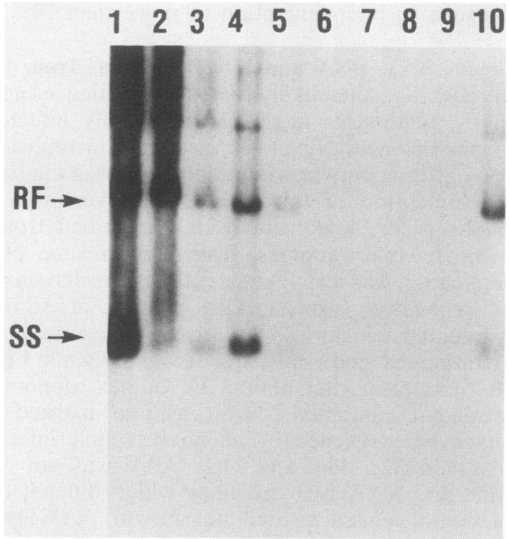

FIG. 2. Hirt supernatants were prepared from infected M104 and 293 cells and analyzed for AAV DNA replication as described in the legend to Fig. 1. Lanes: 1 through 7, DNA derived from M104 cells; 8 through 10, DNA derived from 293 cells; 1 and 8, Ad5 infection; 2, Ad5 and AAV2 coinfection; 3, Ad5*hr*6 infection; 4, Ad5*hr*13 infection; 5, Ad5*sub*316 infection; 6, mock infection; 7 and 9, AAV2 infection.

ern blotting, and hybridization with an AAV-specific probe. In KB cells coinfected with adenovirus and AAV, the predominant 2.3-kilobase AAV transcript is clearly present (Fig. 3, lanes 7, 8, 7a, and 8a), and the larger transcripts, although not well resolved, are also present. There is no evidence of AAV-specific transcription in either the adenovirus-infected KB cells (lanes 5 and 6) or mock-infected C178 cells (lanes 1 and 2). In the adenovirus-infected C178 cells, the AAV-specific transcripts are again apparent (lanes 3 and 4). Much longer exposure of lanes 1 and 2, containng poly(A)$^+$ RNA from two preparations of mock-infected C178 cells, did not reveal any AAV-specific RNA, indicating that the integrated AAV genomes, at least in the C178 cell line, are transcribed at low levels, if at all.

Analysis of cellular DNA in latently infected cells. DNA from latently infected cells was examined to determine whether viral DNA sequences were integrated into cellular DNA as had been reported for the other described latently infected cell lines (5, 14, 21). Initially, cellular DNA isolated from several of the latently infected cell lines was digested with a restriction enzyme that does not cut AAV DNA to determine whether any viral sequences present were contained in high-molecular-weight DNA. The results of digestion with *Bgl*II and *Pvu*II can be seen in Fig. 4. All of the cell lines (except M26, a negative control) contain a limited number of bands which hybridize with AAV DNA. These AAV-specific bands are either the same molecular weight or larger than AAV. The most probable interpretation of the observed patterns is that the viral sequences are integrated into cellular DNA. In addition, in cell line M34, virus-specific sequences that comigrated with adeno-associated viral DNA were observed. This could indicate that in this cell line some sequences are being maintained episomally or alternatively that integrated sequences may occasionally be excised. Free AAV DNA, in addition to integrated viral genomes, was also seen in the latently infected Detroit 6 cells (14). The finding of bands of different apparent molecular weight when the same cellular DNA was restricted with different enzymes argues against the presence of virus-specific sequences in a concatemeric episome composed only of viral sequences. However, it is impossible to rule out the possibility that the viral sequences are present in a high-molecular-weight episome composed of both viral and cellular sequences. In addition, the finding of virus-specific cellular bands with different electrophoretic mobilities after the digestion of several clones with the same enzyme suggests that integration of AAV is not sequence specific with respect to KB cell DNA. A maximal value for the number of separate integrations of AAV sequences can be derived by counting the observed bands. However, the resulting number may be too large since, even tough a large excess of restriction enzyme was used, the viscosity of the digestion mix made it difficult to ensure that complete digestion was achieved. Therefore, it is clear that the number of integration sites is low, and it is possible that there is actually only one integration site per cloned cell line.

TABLE 3. Rescue of infectious AAV from latently infected KB cells

Rescue of:	Cell line							
	KB	M11	M19	M21	M34	M53	M69	M104
AAV DNA[a]	−[b]	+++	++	+	+++	+++	++	++
Infectious AAV[c]	−	+++	−	+	+++	++	++	++

[a] The indicated cell lines were infected with Ad5 at a multiplicity of infection of 10. Hirt supernatants were prepared from infected cultures at 20 h p.i. and analyzed for the presence of AAV DNA as described in Materials and Methods.

[b] Symbols are as defined in Table 2, footnote *a*.

[c] Duplicate cultures of the samples harvested at 20 h p.i. were harvested at 72 h p.i. for a virus yield assay which was performed as described in Materials and Methods.

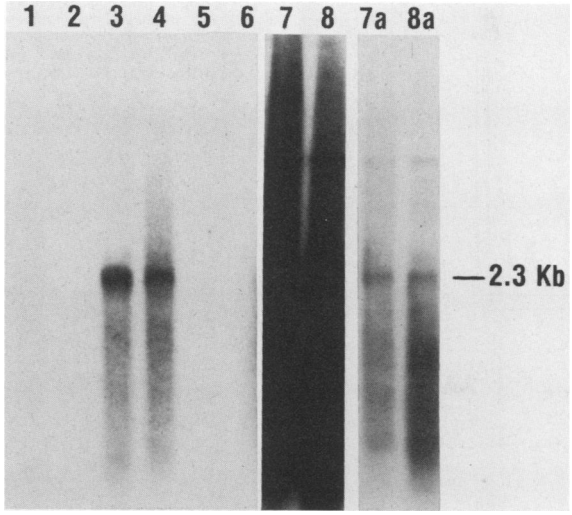

FIG. 3. Poly(A)$^+$ RNA was extracted from infected and control cultures and analyzed as described in Materials and Methods. Consecutive lanes demonstrate RNA from two separate preparations. Lanes: 1 and 2, uninfected C178 cells; 3 and 4, Ad5-infected C178 cells; 5 and 6, Ad5-infected KB cells; 7 and 8, KB cells coinfected with Ad5 and AAV2; 7a and 8a, lighter exposures of lanes 7 and 8.

The DNAs from the latently infected cells were next digested with two multicut restriction enzymes. Digestion with *Hinc*II gave a DNA pattern that showed the presence of the internal fragment A in all cell lines tested and the internal fragment C in four of the six tested (M69, M104, M34, and C178; Fig. 5A). However, the terminal fragments of the viral DNA were not evident. This finding is expected if the viral sequences are integrated. Additional viral sequences are present in bands of greater molecular weight than expected, which might represent the junction fragments of cellular and viral DNA or alternatively result from aberrant viral genomes. Furthermore, all cell lines had a new virus-specific band with the electrophoretic mobility expected from digestion of a head-to-tail concatemer of viral DNA (indicated by the arrow in Fig. 5A).

Restriction digestion of the same latently infected cellular DNAs by *Kpn*I gave similar results (Fig. 5B). The internal viral fragment A was clearly present in all the cell lines tested, and the viral terminal fragments were not, again suggesting that the viral DNA is integrated into the cellular DNA. New virus-specific bands of decreased electrophoretic mobility are evident, which may represent the junctions of viral and cellular sequences. (This is currently being tested directly.) The expected fragment resulting from head-to-tail concatemers of viral DNA would have comigrated with the viral internal fragment A, and therefore the possible presence of concatemers suggested by the *Hinc*II digestion cannot be assessed with *Kpn*I.

In a direct attempt to identify viral head-to-tail concatemers, the same cellular DNAs from the latently infected clones were finally digested with enzymes which cut the viral genome once. The results obtained when the cellular DNAs

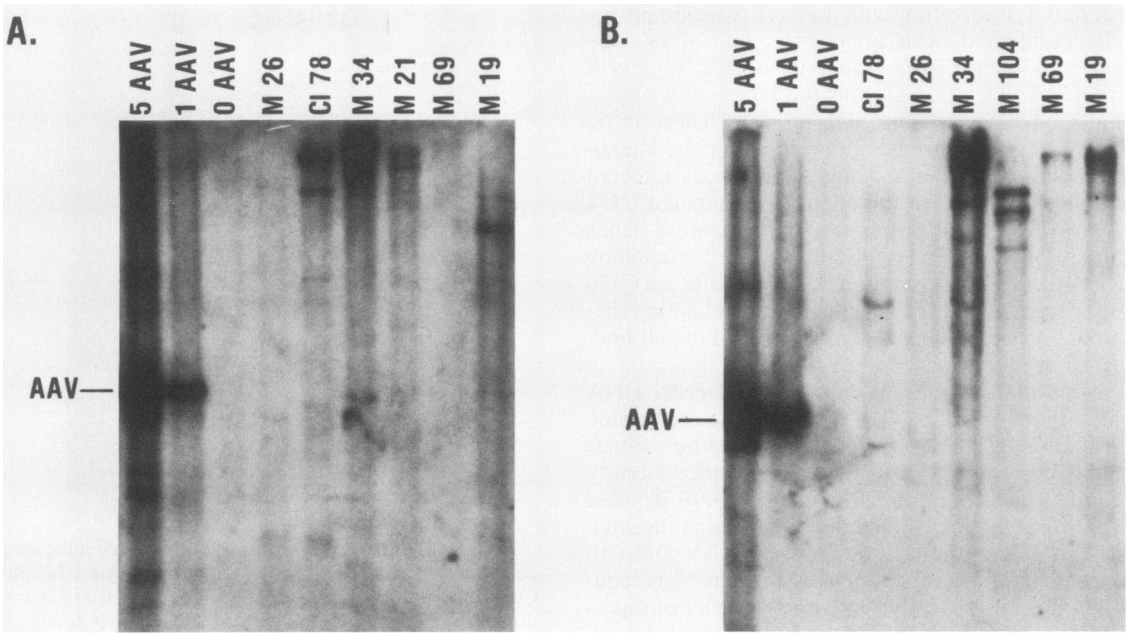

FIG. 4. Cellular DNA (20 μg) was digested with *Pvu*II (A) or *Bgl*II (B), electrophoresed on 0.7% agarose gels, and blotted and hybridized as described in Materials and Methods. Control wells contained 20 μg of calf thymus DNA supplemented with AAV DNA corresponding to 1 to 5 genome equivalents per cell.

were digested with *Hin*dIII are shown in Fig. 6A. Again, the general lack of free viral DNA is evident since there are no clear bands which comigrate with either viral fragment. A virus-specific band that comigrated with viral DNA was clearly evident in three of the five rescuable lines tested (M53, M104, and M34). The pattern seen with C178 DNA is ambiguous, but there is a minor band which may be the result of concatemeric sequences. Cell line M21 does not appear to contain concatemeric viral genomes. M26, a nonrescuable clone, appears to have several faint AAV-specific bands. These are most likely due to a high level of background, since these bands were not reproducibly observed and in this gel there was also a similar amount of hybridization in the control well containing calf thymus DNA without added AAV. It is also formally possible that the faint bands represent low levels of AAV sequences that are not able to be rescued. In a separate experiment, nine cell DNAs from KB cell clones isolated after AAV infection (Table 1) from which AAV DNA was not rescued were shown not to contain AAV-specific sequences (data not shown).

An equivalent experiment shows the pattern resulting from digestion of latently infected cellular DNAs with the restriction enzyme *Bam*HI (Fig. 6B). Again, bands comigrating with viral DNA fragments are not evident, reflecting the lack of free viral genomes. AAV-specific bands comigrating with viral DNA are clearly present in three of the lines (M34, M104, and M69). Again, the pattern observed with C178 is ambiguous. A band is present but appears to have a mobility slightly slower than that of viral DNA. Digestion of M19 DNA resulted in the appearance of an AAV-specific band that migrated faster than did viral DNA and more slowly than did *Bam*HI A. The mobility observed is consistent with the size of the deleted genome that was rescued from M19 cells. Thus, five of the seven lines tested clearly contain concatemeric viral sequences. One of the remaining lines (C178) gave ambiguous results with two enzymes, and the only clear negative, M21, is a line from which AAV DNA is efficiently rescued but infectious virus is only inefficiently rescued. The prevalence of concatemeric sequences suggests three possibilities. One is that infecting viral genomes recombine before integration. Alternatively, the infecting DNA may undergo some replication before integration. The mechanism of this DNA replication must be different from the DNA replication seen in a productive AAV infection, since concatemers formed as part of a productive infection are head-to-head and tail-to-tail (3). The third possibility is that after integration, the viral sequences are amplified. Finally, the high percentage of clones from which AAV DNA can be rescued that clearly contain concatemeric sequences suggests that integration of concatemeric sequences may be a necessary precondition for either integration or rescue. The AAV DNA-positive clones which apparently do not contain concatemers may have undergone deletion or rearrangement not detected at this level of analysis which masks the presence of head-to-tail multiple genomes.

The data derived from the various cell lines have been summarized in Table 4. It can be concluded that latent infection of KB cells with AAV2 involves the integration of AAV sequences into a limited number of sites in the cellular DNA. Superinfection with a helper virus, either adenovirus or HSV, rescues AAV DNA replication and in almost all cases results in the release of infectious virus. In addition, most of the cell lines show clear evidence for integration of head-to-tail concatemers of viral DNA.

DISCUSSION

We demonstrated that the establishment of latent infection of AAV in human cells in vitro is remarkably efficient. Viral DNA replication can be rescued from greater than 10% of KB cell clones derived from cells infected with a high multiplicity of AAV in the absence of a helper virus.

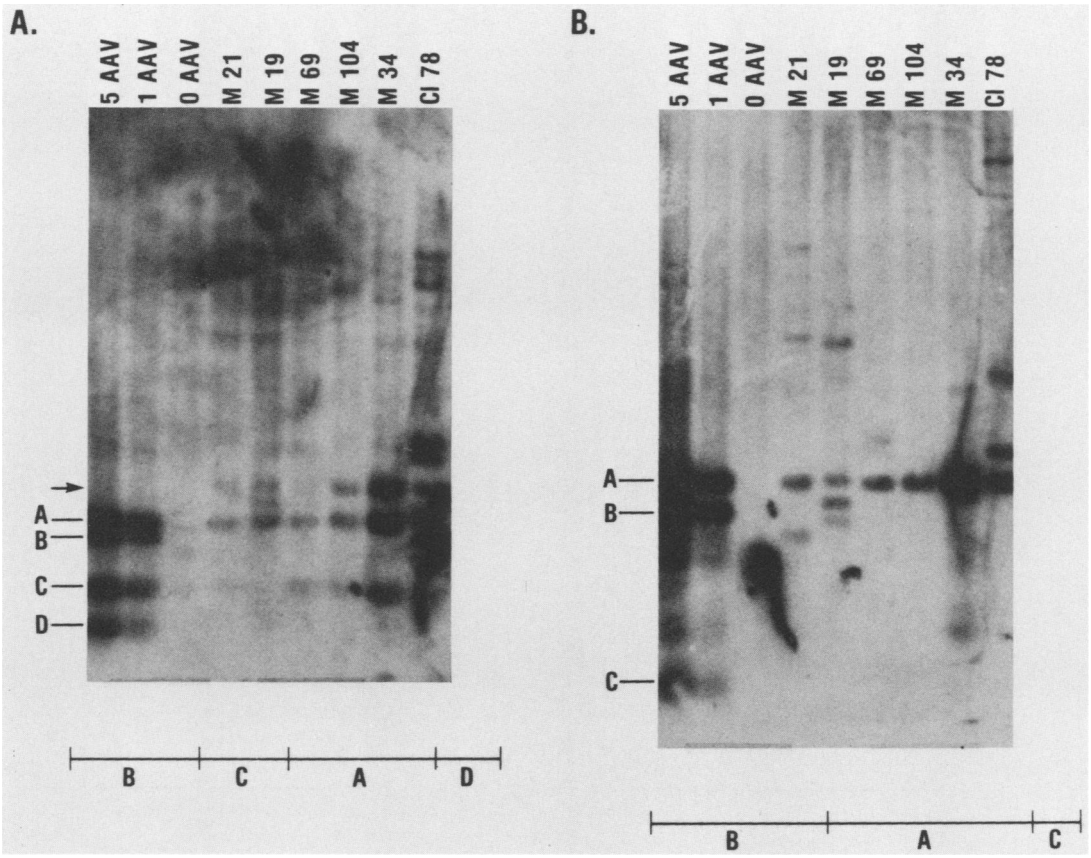

FIG. 5. Same as in the legend to Fig. 4 except that restriction enzymes used were *Hin*cII (A) and *Kpn*I (B).

Efficiencies of 1 to 2% and 30% have been reported by others (14, 21), although in the latter report the cells were cloned 30 passages p.i. and it is remotely possible that the presence of AAV sequences affected the viability of the cells. Since AAV is a prevalent human virus with two-thirds of American adults showing serological evidence of infection (8), its predilection for establishing latency might have consequences for human health. Indeed, there is evidence that AAV does establish latent infections in vivo. A total of 2 to 3% of lots of primary human embryonic kidney cells and 20 to 30% of primary African green monkey kidney cells were shown to be contaminated with AAV (27). It seems probable that integration of AAV DNA in vivo occasionally results in mutation. However, the only reported clinical effects of AAV infection are beneficial rather than pathological. There is a suggestion (36) that concomitant infection with AAV can modulate the severity of a helper adenovirus or herpesvirus infection as has been shown to occur in vitro (42). In addition, AAV infection has an inhibitory effect on turmorigenesis initiated by adenovirus, adenovirus-transformed cells, and HSV-2-transformed cells (6, 18, 31, 41).

The efficiency of the establishment of latent infections by AAV is advantageous for the design of AAV-based integrative vectors and for studies of eucaryotic recombination mechanisms. The ability to integrate the viral genome in 14% of infected cells means that AAV may be a practical integration vector for clinical purposes. AAV-based vectors with large deletions of viral sequences (24, 55; Laughlin, in preparation) transduce foreign genes into human cells, and it is probable that the only viral sequences necessary for integration into cellular DNA are the inverted terminal repeats of 145 nucleotides. In addition, integration of AAV DNA occurs in the absence of helper virus and without detectable expression of the AAV genome (4). Therefore, the integration of viral DNA must be accomplished by cellular mechanisms, and AAV may be a valuable probe for the study of eucaryotic genetic recombination.

The ability of the AAV genome to be rescued from the latent state provides an exploitable feature for basic research. AAV-based vectors which transduce the *neo* gene at a maximum frequency of 10% can be rescued from transduced cells after helper virus superinfection (24, 55). Libraries of cellular genomes could be made with AAV vectors and transfected into mammalian cells. After positive selection, individual cell genes with their natural promoters coding for selectable functions could be isolated by adenovirus superinfection of positive cell clones.

It is probable that no additional helper virus functions, other than those necessary for AAV replication, are required for rescue of the integrated genome. However, our results with Ad5*hr*6, which is deficient in normal E1B 55K protein synthesis, together with previously reported results in latently infected Detroit 6 cells (40), suggest that this protein may influence the efficiency of rescue. A functional relationship between AAV replication and the E1B 55K protein was strongly suggested by the report that AAV infection of an adenovirus-transformed hamster cell line resulted in an 80% decrease in immunoprecipitable E1B protein (41). This relationship is further supported by the report that the E1B 55K

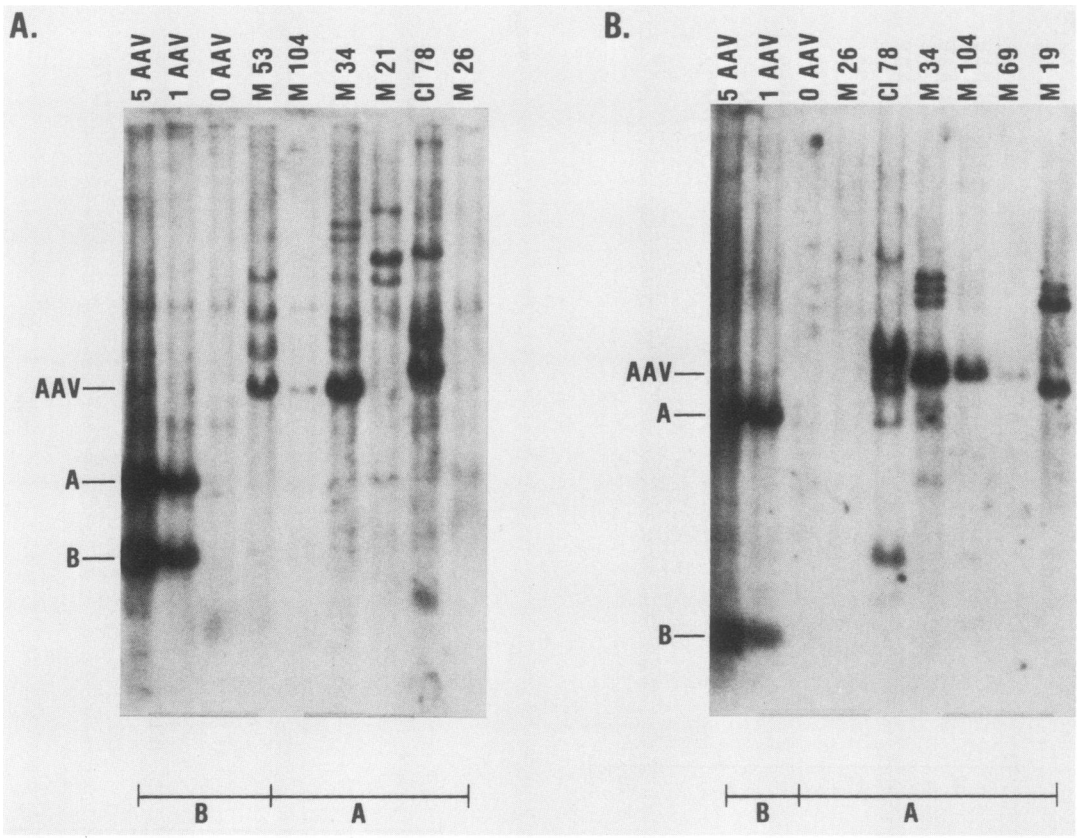

FIG. 6. Same as in the legend to Fig. 4 except that restriction enzymes used were *Hin*dIII (A) and *Bam*HI (B).

protein is physically associated with the E4 25K protein in productively infected cells (49). Both of these proteins have been demonstrated to function in the helper activity provided by adenovirus for AAV replication, although the E4 protein has a more direct role (32, 45, 46). In addition, there is an indication that AAV replication (10) and rescue from latently infected cells (data not shown) are more efficient in rapidly growing cells. Therefore, the finding that the adenovirus E1B 55K protein can form complexes with the cell cycle regulatory protein p53 in adenovirus-transformed cells (50, 57) further suggests that this cellular protein may influence the efficiency of rescue. It is not clear whether rescue occurs as a result of replication of the integrated genome or of spontaneously excised genomes. However, since rescue is efficient, occurring in 20 to 50% of the cells as determined by an indirect immunofluorescent assay of AAV capsid antigens in latently infected cells after superinfection with adenovirus (data not shown), and since an analysis of AAV sequences in latently infected cells did not provide evidence for much free viral DNA, the former hypothesis seems more probable. It has been suggested that AAV replication is more efficient when the infected cells are rapidly growing (10), and an analogous effect has been noted with the rescue of AAV from latently infected cells (data not shown). This suggests that replication of an integrated genome in the presence of helper virus may result in discrete replication and release of the AAV genome. It is not yet clear whether every integrated AAV genome can be rescued. The high percentage of latently infected clones which contain head-to-tail concatemers suggests that concatemer formation may be necessary for subsequent rescue. However, there are two clones, M21 and C178, which do not appear to contain concatemers, although more detailed mapping of their integrated sequences will have to be done to prove the absence of concatemers since other small mutations or rearrangements may prevent the recognition of a concatemer at this level of analysis.

In the one latently infected clone carefully examined, there was no evidence for expression of the integrated AAV genomes in the absence of helper virus coinfection. Similarly, there was also no evidence for AAV expression in latently infected Detroit 6 cell lines (29) or KB cell lines latently infected with AAV1 (21). This can probably be

TABLE 4. Characteristics of KB cell clones latently infected with AAV2

Cell line	Rescue of infectious virus	No. of inserts[a]	Intact internal sequences	Concatemers
C178	+ +[b]	1–4	+	?[c]
M11[d]	+ + +	1–2	+	+
M19	−	1–3	−	+
M21	+	1–4	−	−
M34	+ + +	1–6	+	+
M53	+ + +	1–2	+	+
M69	+ +	1–2	+	+
M104	+ +	1–2	+	+

[a] This figure was derived by counting the number of bands repeatedly observed with no-cut enzymes and half the number of bands observed with one-cut enzymes (excluding any bands that comigrating with AAV and were presumably derived from concatemeric sequences).

[b] Symbols are as defined in Table 2, footnote *a*.

[c] ?, Not known.

[d] M11 was analyzed more recently than the other cell lines, and although the data are not shown, they have been summarized.

explained by the recent finding of two groups (N. Muzyczka, unpublished data; B. J. Carter, unpublished data) that expression of the left open reading frame of AAV which encodes the *rep* gene may be lethal to some host cells. The *rep* gene has been shown to be essential for AAV DNA replication (23, 54). Alternatively, expression of the capsid proteins encoded by the right open reading frame may be deleterious to cell growth, as has been observed with expression of the capsid proteins of the autonomous parvovirus minute virus of mice (44). It is well documented that the promoter for the AAV capsid proteins, p40, can function in the integrated state to express nonviral genes such as the *neo* gene which provides resistance to the aminoglycoside G418 (55).

The examination of the pattern of viral DNA integration in the latently infected cells allows several conclusions. The conclusion that the viral sequences are integrated into cellular sequences is strongly supported by the slow electrophoretic mobility of virus-specific sequences after digestion with restriction enzymes that do not cut AAV DNA and by the absence of viral terminal fragments after digestion with multicut enzymes. Digestion with an enzyme that did not cut viral sequences also indicated that there were a limited number of integration sites (Table 4), and the sites appeared to be different in each line since the mobility of the virus-specific fragments varied from one cell line to another. Digestions with restriction enzymes that cut near the viral termini (*Sma*I and *Nco*I, data not shown) show release of the expected large internal fragment. These results are consistent with the previous report that integration is virus sequence specific and involves the covalent joining of the viral termini to cellular sequences. There is, as yet, no evidence for any specific cellular nucleotide sequence required for AAV integration. These data also suggest that integration of viral DNA usually occurs without much rearrangement. However, it is possible that some rearrangement did occur, since the rescued AAV DNA of one of the latently infected cell lines contained a deletion and adenovirus superinfection of this cell line did not result in the production of infectious virus. Defective AAV virions containing genome deletions are normally produced during AAV replication (17). The viral stock used to initiate the latently infected cells was equilibrium banded in CsCl to eliminate defective virions with large deletions, but virions containing small deletions do not differ enough in density to be separable. Therefore, it is possible that cell line M19 was initially infected with a deleted genome. M19 appears to have at least two sites of integration, yet only one class of deleted DNA was rescued from these cells. This implies either that the viral sequences in one integration site are not rescuable or that the integrated sequences were amplified and some were transposed to another site in the cellular DNA. It is evident that the examination of human cells latently infected with AAV will provide important information about eucaryotic recombination and that the next stage (in progress) is the molecular cloning of the integrated AAV sequences from the latently infected cells.

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