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Integration of the Adeno-Associated Virus Genome into Cellular DNA in Latently Infected Human Detroit 6 Cells

ANDREW K. M. CHEUNG,[1] M. DAVID HOGGAN,[2] WILLIAM W. HAUSWIRTH,[1] AND KENNETH I. BERNS[1]*

Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida 32610,[1] and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205[2]

A clone of human cells (Detroit 6) latently infected by adeno-associated virus (AAV) has been characterized with regard to the status of the viral DNA. In both early (9 to 10) and late (118) passages of the clone, AAV DNA was recombined with host DNA, at least in some cases as a head-to-tail tandem repeat, via the terminal sequences of the viral genome. However, it was not possible to distinguish between integration into chromosomal DNA and very large plasmids ($>20 \times 10^6$ molecular weight) which contain both viral and cellular DNA sequences. Although evidence for some modifications of the viral sequence was obtained, most of the integrated sequences appeared to be intact. In some cases sequences of undetermined origin separated adjacent copies of the viral genome. Free copies of the AAV genome were detectable in late passage cells, but not in early passage cells. The orientation of nucleotide sequences present in the free AAV DNA from late passage cells was indistinguishable from that of virion DNA. With one notable exception, the organization of the integrated AAV sequences as determined by restriction enzyme digestion remained constant with continued passage. Digestion with *Sma*I, which cleaves within the palindromic region of the terminal repetition in AAV DNA, produced reproducibly different patterns when early and late passage DNAs were compared. Several models for rescue of free copies of the genome from the integrated DNA are possible, all of which involve the terminal repetition.

Adeno-associated virus (AAV) is a defective parvovirus which requires coinfection with an adenovirus helper for a productive infection (1, 16). In the absence of helper coinfection, AAV penetrates to the nucleus where the virion is uncoated (23; Berns and Adler, unpublished data). Under these conditions, no AAV-specific macromolecular synthesis is detectable, but the AAV genome may form a stable relationship with the cell, and infectious AAV can be induced by subsequent infection with adenovirus as first reported by Hoggan et al. (17). These investigators found that up to 2% of primary human embryonic kidney (HEK) cell lots and 20% of African green monkey kidney (AGMK) cell lots would yield AAV after infection with purified adenovirus, although the cells were negative for AAV antigens before addition of the helper.

The cryptic infection in vivo can be mimicked in tissue culture. A continuous line of human cells (Detroit 6) (3) infected at high multiplicity (250 50% tissue culture infectious doses per cell) continued to release AAV upon addition of adenovirus for over 100 passages, although no AAV antigens could be detected in the absence of added adenovirus (5). After 39 passages the cells were cloned, and 18 of 63 clones tested were able to produce AAV-2 when challenged with adenovirus. One of the positive clones was then recloned. All of the recloned populations were capable of being induced by adenovirus to produce AAV. Two of these clones remained positive for AAV for over 67 passages. By reassociation kinetics they contained three to five copies of the AAV genome per diploid amount of cell DNA.

These results have been confirmed and extended by Handa et al. (12). Human KB cells latently infected with AAV-1 contained four to six copies of AAV DNA per cell. Cell DNA to be assayed by reassociation kinetics was fractionated into chromosomal and nonchromosomal DNA by the network technique (26). The network DNA contained 80% of the AAV sequences, and it was concluded that the AAV DNA was integrated into the host genome. Because 20% of the AAV sequences were not associated with the network, it was also possible that some of the AAV DNA existed as a plasmid.

In this paper we report on the organization of AAV nucleotide sequences in a clone of latently infected Detroit 6 cells at both early (9 to 10)

and late (118) passages. Multiple copies of AAV DNA appear to be incorporated into the cellular DNA, with evidence for some head-to-tail tandem repeats which remain constant for over 100 passages. However, by passage 118 free AAV DNA which is indistinguishable from virion DNA is also present in the cells.

MATERIALS AND METHODS

Cell lines. The origin and properties of the latently AAV-2-infected Detroit 6 cells have been described by Berns et al. (5). B6240 is the parental line from which the AAV latently infected cells B7374IIID5 were derived. B6240 cells were infected with AAV-2 (250 50% tissue culture infectious doses per cell). At passage 39, the culture was cloned. One of the positive clones was recloned ($B7374IIID_5$, hereafter referred to as D5).

Viruses. The growth and purification of AAV-2 (AAV 2H) and adenovirus type 2, used as helper, have been previously described (8, 11).

Extraction of viral and cellular DNAs. Viral and cellular DNAs were prepared as described previously (5, 8, 11, 13).

Restriction enzymes. *Hpa*I, *Bgl*II, *Hin*dIII, *Bam*HI, *Hae*III, *Hpa*II, *Hin*cII, and *Sma*I were purchased from Bethesda Research Labs. *Bal*I and *Pvu*I were purchased from New England Biolabs.

All enzyme digestions were carried out at 37°C for 4 to 6 h with 1 U of restriction enzyme per μg of DNA. The buffer used for *Hpa*I was 20 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, and 6 mM KCl; the buffer used for *Bam*HI, *Bal*I, and *Bgl*II was 10 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, and 7 mM 2-mercaptoethanol; the buffer used for *Hin*dIII and *Hin*cII was 10 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, and 60 mM NaCl; the buffer used for *Pvu*I was 6 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 150 mM NaCl; the buffer used for *Hpa*II and *Hae*III was 10 mM Tris-hydrochloride (pH 7.5) and 6 mM $MgCl_2$; and the buffer used for *Sma*I was 15 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, and 15 mM KCl.

Agarose gel electrophoresis. Restriction digests of cell DNA (10 μg) were electrophoresed in 1% agarose vertical slab gels (34 by 17 by 0.3 cm) with buffer containing 40 mM Tris-hydrochloride (pH 7.8), 1 mM EDTA, and 5 mM potassium acetate at room temperature.

As a control, AAV-2 virion DNA was mixed with carrier calf thymus DNA (Sigma) to compensate for possible concentration effects on DNA migration.

Transfer of DNA onto nitrocellulose filters. After electrophoresis, DNA fragments in the gel were denatured in situ by immersing the gel in 500 ml of 1 M KOH for 10 to 20 min at room temperature. The gel was neutralized by adding an equal volume of 1 M Tris-hydrochloride (pH 7)–1 M HCl for 30 to 60 min and then washing with 500 ml of 6 × SSC (SSC, 0.15 M NaCl–0.015 M sodium citrate), pH 7, for 10 to 20 min. The DNA was transferred to a nitrocellulose filter (Schleicher-Schuell, type BA85, 0.45-μm pores) as described by Southern (24). Briefly, the gel was laid on a wick made of Whatman no. 1 paper. The wick was supported by a glass plate with two sides extending over the edges of the glass plate into a trough of 6 × SSC below. Immediately on top of the gel were placed the nitrocellulose filter, a sheet of Whatman no. 3 paper, and a stack of Kim-wipes. DNA transfer was carried out overnight with several changes of the Kim-wipe paper. The nitrocellulose filter was then washed in 2 × SSC, pH 7, for 10 to 15 min, blotted dry, and baked for 2 h at 80°C in a vacuum oven.

Nick translation of AAV DNA. The nick translation procedure of Kelly et al. (18) and Rigby et al. (22) was employed. The reaction mixture (1 ml) contained 50 mM Tris-hydrochloride (pH 7), 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.05 mg of bovine serum albumin, 0.1 mM each of dCTP, dATP, and TTP, 3 nM of [α-^{32}P]dGTP (specific activity, 250 to 350 Ci/mmol, Amersham), 15 to 30 U of *Escherichia coli* polymerase I (gift of T. Kelly), 0.01 μg of pancreatic DNase I, and 5 μg of duplex AAV DNA. The reaction was carried out at 15°C for 45 to 50 min, stopped by addition of EDTA to 20 mM, and extracted twice with phenol and then ether. The aqueous phase was passed through a Sephadex G-50 column (1 by 10 cm, Pharmacia Fine Chemicals), and the first radioactive peak was collected. A specific activity of 10^7 to 10^8 cpm/μg was achieved in the probe.

Filter hybridization and autoradiography. All steps in the filter hybridization were carried out at 37°C by a modification of the procedure described by Denhardt (10). Baked nitrocellulose filters were presoaked in 5 × SSC containing 0.08% bovine serum albumin, 0.08% Ficoll, 0.08% polyvinylpyrollidone, 50% formamide, 20 mM Tris-hydrochloride (pH 7.4), and 0.5% sodium dodecyl sulfate for 30 min. The filters were allowed to anneal in an identical solution containing 10 ng of alkaline-denatured radioactive probe per ml and 50 μg of tRNA per ml for 24 to 96 h. After hybridization, the filters were rinsed and washed with the hybridization buffer for 30 min. The filters were then rinsed and washed in 2 × SSC (pH 7) for 30 min, air dried, and autoradiographed with Cronex 4 X-ray film (Dupont) in conjunction with Kodak intensifier screens for 4 days to 2 weeks at −70°C.

RESULTS

Detection of AAV nucleotide sequences in Detroit 6 cell DNA. By using the Southern blotting technique (24), AAV nucleotide sequences could be detected in DNA isolated from the D5 clone of latently infected Detroit 6 cells. AAV-specific sequences were present in slowly migrating and presumably high-molecular-weight DNA isolated from cloned D5 cells at both early (passage 9 to 10) and late (passage 118) passages (Fig. 1). No AAV sequence homology, however, could be detected in control Detroit 6 cell DNA. In the DNA from late passage cells, an additional band could be detected which corresponded in mobility to free linear duplex DNA. To assess in greater detail the absence of low-molecular-weight AAV DNA in early passage cells, the supernatant and pellet fractions of a Hirt (15) extract were assayed for

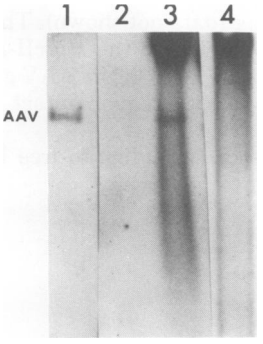

FIG. 1. *Detection of AAV-2-specific sequences in latently infected Detroit 6 cells. Total cellular DNAs were fractionated by electrophoresis in 1% agarose slab gels. The DNAs were denatured and transferred to nitrocellulose filters by the blotting procedure (24). Viral sequences were detected by DNA-DNA hybridization followed by autoradiography. See text for details. Lane 1, 10^{-5} μg of AAV-2 DNA with 10 μg of calf thymus DNA; lane 2, Detroit 6 DNA; lane 3, D5 DNA at passage 118; and lane 4, D5 DNA at passage 9 to 10.*

AAV sequences. Presumably, free AAV DNA of unit length would be in the supernatant fluid as is found with normally infected cells. However, when equal amounts of DNA (10 μg) isolated from both the supernatant and pellet fractions were tested by the blotting technique, AAV sequences could only be detected in DNA isolated from the pellet (Fig. 2). Reproducible patterns were obtained when the DNA from the pellet was digested with either *Hin*dIII or *Bam*HI (one site in virion DNA), but none of the species produced comigrated with fragments produced by *Hin*dIII or *Bam*HI digestion of virion DNA.

Integration of AAV DNA. The slowly migrating species of AAV sequences in Fig. 1 could represent either a plasmid containing repeated copies of AAV DNA or, alternatively, AAV sequences integrated into host DNA. To test the possibility of integration, cellular DNA was digested with three enzymes which do not cleave AAV DNA. An increased mobility of the AAV sequences after digestion would indicate association with cellular sequences. Two of three enzymes tried (*Bgl*II and *Hpa*I) clearly increased the mobility of the AAV sequences and resulted in sharper bands (Fig. 3). Digestion with *Pvu*I did not do so. These data suggest that AAV sequences are associated with cellular sequences. To account for the *Pvu*I data, we suggest that the distance between the *Pvu*I sites bounding the AAV sequences is greater than the average size of fragments containing AAV sequences in the undigested DNA. The data (not shown) obtained by digestion of DNA from early passage cells were qualitatively identical. We could not resolve more than one band in any of the digests in Fig. 3, even when the samples were analyzed on 0.3% agarose gels. Despite the difficulty in resolving different species in this molecular weight range, these data are consistent with the possibility that several copies of the DNA may integrate in a cluster.

Integration takes place at the termini of the AAV genome. As isolated from the virion AAV DNA is linear. Recombination with cellular sequences might occur via either a linear or a circular intermediate. Only one recombina-

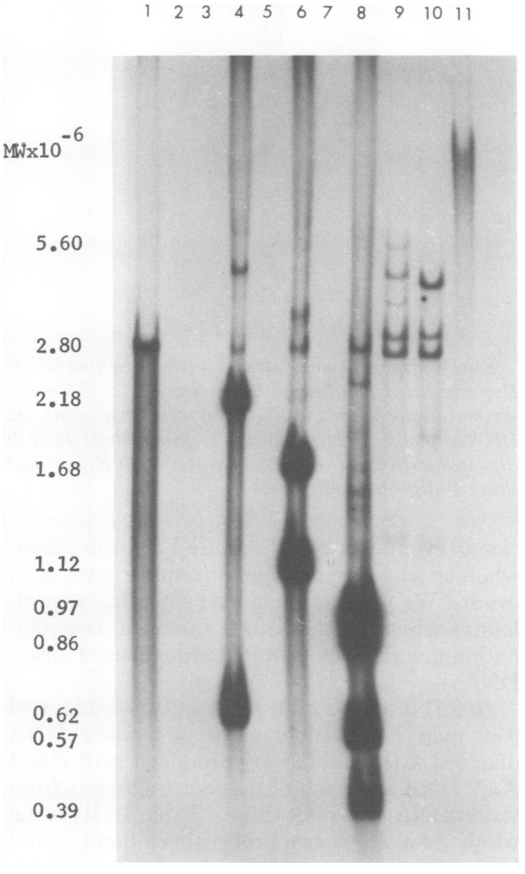

FIG. 2. *Analysis of D5, passage 9 to 10, DNA extracted by the method of Hirt (15). A 10-μg amount of DNA was electrophoresed in each slot, blotted, hybridized to ^{32}P-labeled virion DNA, and autoradiographed. Lanes 1, 4, 6, and 8 contain 10^{-5} μg of AAV-2 DNA and 10 μg of calf thymus DNA. Lane 1, undigested AAV-2 DNA; lane 4, cleaved with BamHI; lane 6, cleaved with HindIII; and lane 8, cleaved with HincII. Lanes 2, 3, 5, and 7 are D5 Hirt supernatant fluid DNA. Lane 2, undigested; lane 3, cleaved with BamHI; lane 5, cleaved with HindIII; and lane 7, cleaved with HincII. Lanes 9, 10, and 11 are D5 Hirt pellet DNA. Lane 9, cleaved with HindIII; lane 10, cleaved with BamHI; and lane 11, undigested.*

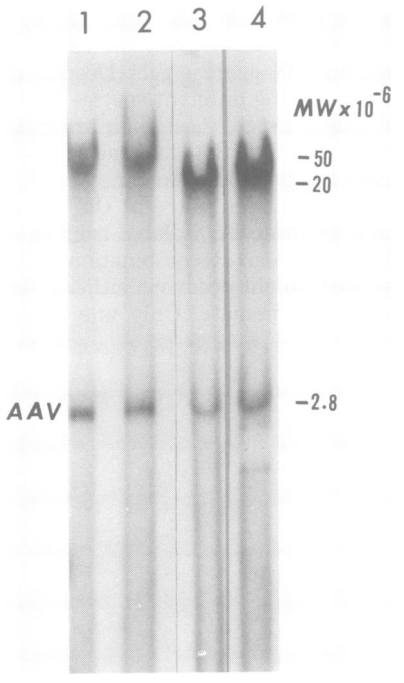

FIG. 3. *Integration of viral sequences in host DNA. Experimental conditions were similar to those described in the legend to Fig. 1. In this experiment, D5 DNA at passage 118 was (lane 1) undigested; (lane 2) digested with PvuI; (lane 3) digested with BglII; and (lane 4) digested with HpaI.*

tional event would be required for the latter, whereas the former would require two such events. We present evidence below that regardless of which mechanism is involved, recombination involves the terminal sequences of virion DNA.

*Hinc*II cleaves virion DNA at 0.307, 0.51, and 0.86 map units. Blots of early passage DNA digested with *Hinc*II were produced with intact AAV DNA and each of the four fragments from virion DNA as probes (Fig. 4, Table 1). By using whole AAV DNA as a probe, seven bands could be detected (one to seven in terms of increasing mobility). Two bands (bands 5 and 7) corresponding in mobility to the internal fragments (A and C) were specific for A and C sequences, respectively. A third band (3) contained both A and C sequences and migrated as an AC fusion product (i.e., its molecular weight = A + C). Thus, in some of the integrated DNA the *Hinc*II-A/C cleavage site was lost. Whatever modification caused this could not have been too extensive, because with a similar digestion by *Pst*I (cleavage at 0.08, 0.42, 0.92) all of the A sequences (0.42–0.92) were associated with a band comigrating with A marker and all B sequences (0.08–0.42) were in a band comigrating with B marker (data not shown). Thus, with the exception of the loss of the *Hinc*II-A/C site, all the internal sequences of the AAV genome from 0.08–0.92 appear to remain intact and contiguous.

No bands corresponding to free B- or D-ter-

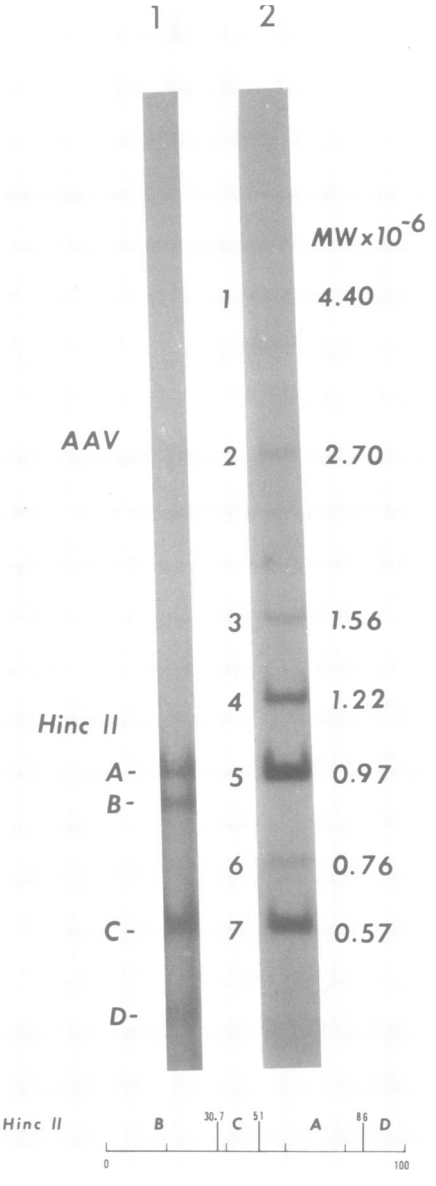

FIG. 4. *Analysis of AAV-2-specific sequences in D5 (passage 9 to 10) DNA after cleavage with HincII. ^{32}P-labeled probe representative of the entire AAV-2 genome was used for hybridization after the blotting procedure. Both AAV-2 virion DNA and D5 (passage 9 to 10) DNA were digested with HincII. At the bottom of the figure is a HincII cleavage map of AAV-2 virion DNA.*

TABLE 1. *Mapping of integrated AAV-2 sequences by HincII digestion*

*Hinc*II species	Probe[a] (in map units)					
	0–30.7	30.7–51.0	51.0–86.0	86.0–100.0	20.0–30.7	42.0–92.0
1	+				+	
2	+			+	+	+
3		+	+			+
4	+			+	+	+
5			+			+
6	+				+	
7		+				+
8[b]				+		+

[a] Probe: 0–30.7 and 20.0–30.7 hybridize to *Hinc*II-B; 30.7–51.0 hybridizes to *Hinc*II-C; 51.0–86.0 hybridizes to *Hinc*II-A; 86.0–100.0 hybridizes to *Hinc*II-D; 42.0–92.0 hybridizes to *Hinc*II-A, -C, and -D.

[b] A faint band which is visible on some of the blots and migrates slightly slower than the *Hinc*II-D marker.

minal fragments could be detected in the *Hinc*II digest. However, two fragments (fragments 2 and 4) containing both B and D sequences were present. The mobility of the smaller fragment containing both B and D sequences corresponded to the size expected for a BD fusion product, suggestive of a head-to-tail tandem repeat. Because fragment 2 is larger than expected for a simple BD fusion product, the additional sequences might represent either cellular DNA or an amplification of the B or D viral sequences. Two fragments (fragments 1 and 6) contained only B sequences. In some blots a very faint fragment containing only D sequences which migrated slightly slower than D marker could also be detected. Because the bands which presumably represent the joining between virion and cellular DNAs contained only terminal sequences of the AAV genome, integration must take place via recombination involving the terminal sequences of AAV DNA. If the recombination intermediate is linear, recombination is via the physical ends. If the intermediate is circular, then recombination in the clone was sequence specific with regard to AAV DNA.

Tandem repeats in the integrated DNA. The data obtained with enzymes which do not cleave AAV virion DNA (Fig. 3) indicate that the several copies of AAV DNA in this clone are integrated at a few sites or possibly only one site in the cell DNA. For the latter to occur, the genome would have to be tandemly repeated. The data presented above for *Hinc*II digestion are consistent with a head-to-tail tandem repeat (e.g., the fragments containing B and D sequences). To gain confirmation of the existence of a tandem repeat of the integrated AAV sequences, cellular DNA was digested with enzymes which have a single site in virion DNA. Two such enzymes were used for this analysis, *Bam*HI (cleavage site at 0.22) and *Hin*dIII (cleavage site at 0.40). *Bam*HI digestion produced five species from early passage DNA (Fig. 5a, lane 3) and seven species from late passage DNA (Fig. 5a, lane 2). The two additional species corresponded to fragments produced by digestion of virion DNA and presumably were derived from the free AAV DNA observed in undigested preparations of DNA from late passage cells (Fig. 1). *Hin*dIII produced six fragments from early passage DNA (Fig. 5b, lane 3) and eight from late passage DNA (Fig. 5b, lane 2). Again, the two extra fragments observed in late passage DNA corresponded to those produced by digestion of virion DNA. Digests of late passage DNA were then hybridized with probes specific for sequences either to the left or right of the cleavage site in virion DNA (Fig. 6). There were three species (species 2, 3, and 5) produced by *Bam*HI which contained sequences both to the left and right of the cleavage site in virion DNA. Species 3 moved very slightly faster than unit length AAV DNA, corresponding to a molecular weight of 2.7×10^6 (suggesting a simple tandem repeat). The other two species (species 2 and 5) had calculated molecular weights of 2.9×10^6 and 1.85×10^6. The relatively low molecular weight of the latter species suggests a large deletion in this integrated sequence. Two additional species, 1 and 6, produced by *Bam*HI digestion contained only sequences either from the left end or the right end of AAV DNA and presumably represent viral sequences joined to cell DNA.

Three species produced by digestion with *Hin*dIII (species 1, 2, and 5; molecular weights 5.1×10^6, 4.2×10^6, and 2.7×10^6) contained sequences both to the left and right of the cleavage site in virion DNA. Hybridization of the right-end probe to band 1 was reproducibly positive, but faint, suggesting the possibility that only a small proportion of sequences to the right of the cleavage site are present in band 1. Two species (species 3 and 7) contained sequences only to the left of the cleavage site and one (species 4) contained only sequences to the right. However, species 4 is an intense band on the autoradiograph and may represent more than one species.

In conclusion, the data from digestion with one-cut enzymes are compatible with (i) integration of AAV sequences, (ii) simple head-to-tail tandem repeats of the integrated AAV sequences, and (iii) other more complex arrangements.

Nucleotide sequence orientation of the free AAV DNA in late passage cells. As shown in Fig. 2 and 5, digestion of early passage

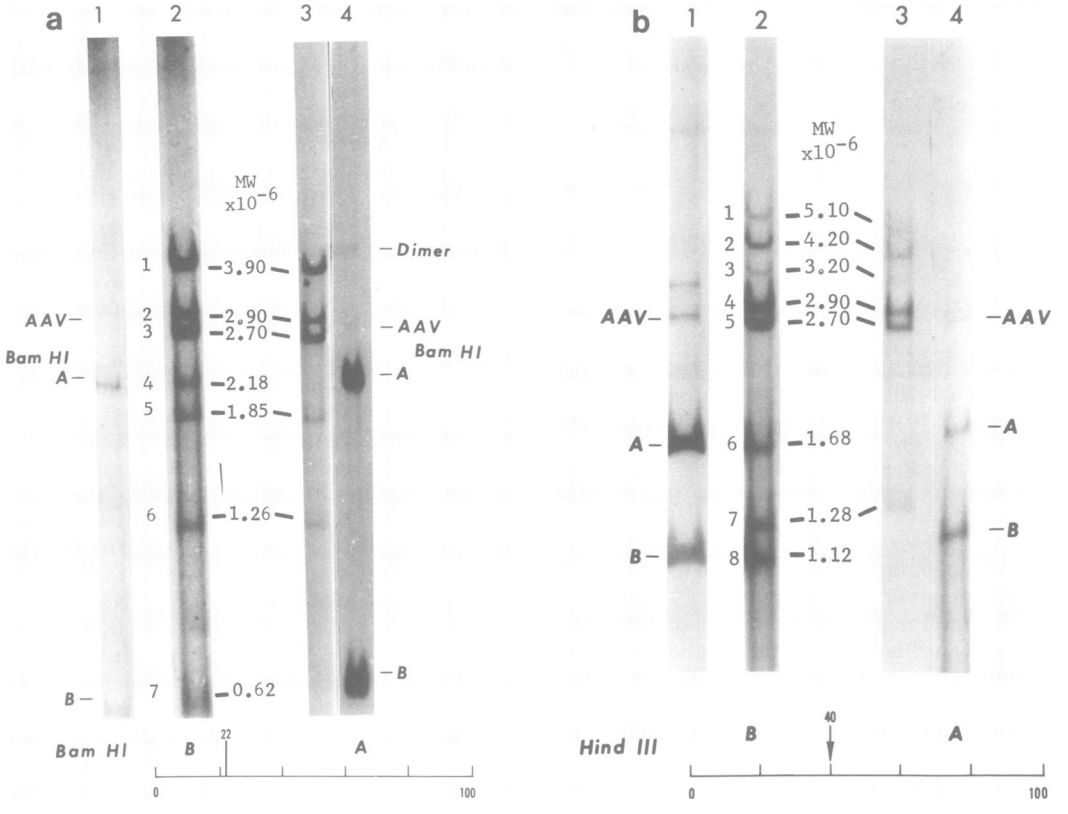

FIG. 5. *(a) Viral sequences in latently infected cell DNA cleaved with BamHI. ^{32}P-labeled DNA representative of the entire AAV-2 genome was used for hybridization. Lanes 1 and 4 are AAV-2 DNA digested with BamHI. Lanes 2 and 3 are D5 (passage 118) and D5 (passage 9 to 10) DNA digested with BamHI, respectively. (b) Analysis of viral sequences in latently infected cell DNA cleaved with HindIII. ^{32}P-labeled AAV-2 virion DNA was used for hybridization. Lanes 1 and 4 are intact plus HindIII-digested AAV-2 DNA. Lanes 2 and 3 are D5 (passage 118) and D5 (passage 9 to 10) DNA digested with HindIII, respectively.*

DNA with single-site enzymes did not yield fragments corresponding to those produced by digestion of virion DNA. However, fragments with those mobilities were produced when free DNA from late passage cells was digested with single-site enzymes, and the nucleotide sequence orientation was the same as that of virion DNA. To check this tentative conclusion, probes specific for sequences either to the left or right of the cleavage site in virion DNA were used. As expected, the additional fragment corresponding in size to the left end contained only sequences specific to the left part of the molecule (Fig. 6a). The same was true for the putative right-end fragment (Fig. 6b). Thus, free AAV DNA which is equivalent to virion DNA in the arrangement of its sequences is generated in late passages of the clone.

Stability of integrated sequences. The stability of the integrated sequences was assessed by comparing the patterns produced by digestion of DNA from early and late passage cells with restriction endonucleases with either one (*Bam*HI, *Hin*dIII, *Sal*I), two (*Eco*RI) or three (*Hin*cII, *Pst*I) sites in virion DNA. In all cases the species derived from integrated sequences remained constant (data not shown except for *Bam*HI and *Hin*dIII, Fig. 5). AAV DNA has an inverted terminal repetition of 145 nucleotides. The first 125 nucleotides have a palindromic sequence which contains two *Sma*I sites near the middle of the palindrome. Two species produced by *Sma*I digestion of early passage DNA were absent in late passage DNA, and two new species of higher molecular weight appeared (Fig. 7). A *Bal*I site is present at nucleotide 122 near the end of the palindrome. Early and late passages gave the same pattern after *Bal*I digestion (Fig. 7). Thus, the only sites on the AAV genome that were detectably altered with continued passage are those found within the palindromic region of the terminal repetition. The

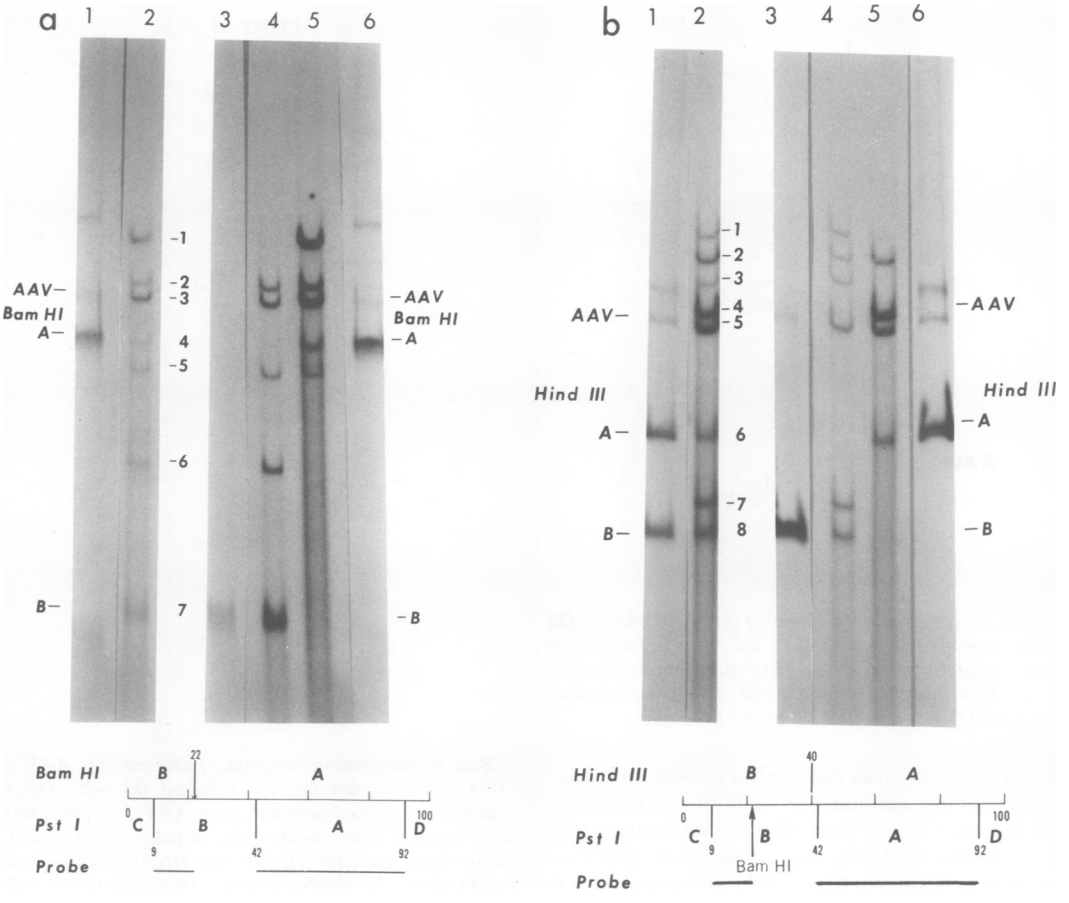

FIG. 6. *(a) Analysis of BamHI digests of AAV and D5 (passage 118) DNAs with probes specific for either side of the cleavage site. See text for details. (Lane 1) AAV and (lane 2) D5 DNA digests were hybridized with complete AAV probe; (lane 3) AAV and (lane 4) D5 DNA digests were hybridized with the left-hand probe; and (lane 5) D5 and (lane 6) AAV DNAs were hybridized with the right-hand probe. (b) Analysis of HindIII digests of AAV and D5 (passage 118) DNAs with probes specific for either side of the cleavage site. See text for details. (Lane 1) AAV and (lane 2) D5 DNA digests were hybridized with entire AAV probe; (lane 3) AAV and (lane 4) D5 DNA digests were hybridized with the left-hand probe; and (lane 5) D5 and (lane 6) AAV DNAs were hybridized with the right-hand probe.*

multiple bands observed with these two enzymes presumably result from association of AAV DNA with cellular sequences in various arrangements or from modifications of the integrated viral genome (either deletion of sites or amplification of other regions of the genome).

Genome structure of rescued virions. Rescue of AAV from latently infected cells is very efficient. Under appropriate conditions, up to 10% latently infected cells can be induced to produce AAV upon addition of helper adenovirus (12). By restriction enzyme analysis, we have compared the DNA isolated from rescued virions with that from virions produced by lytic infection. *Hpa*II cleaves AAV DNA at more than 20 sites. The fragment pattern produced by *Hpa*II cleavage of DNA from rescued virions is indistinguishable from that obtained from normal virions (Fig. 8). Finally, the terminal nucleotide sequences determined by the Maxam-Gilbert technique (20) were also identical (data not shown). By these criteria the genome of rescued virions, like that of free DNA in late passage cells, is indistinguishable from that in virions resulting from lytic infection.

DISCUSSION

In this paper we have characterized the AAV-specific DNA sequences found in a clone of latently infected human cells. From the data several points can be made concerning the state of the viral sequences. In early passages all of

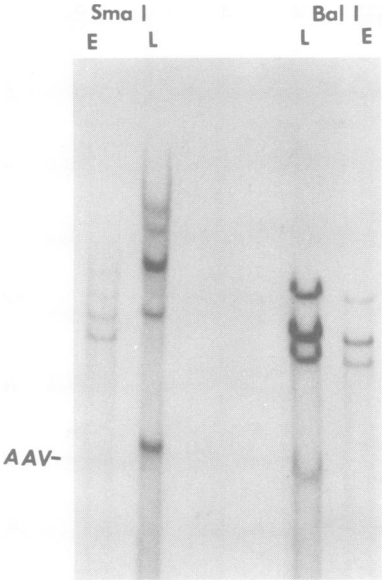

FIG. 7. *Integration pattern of AAV-2 DNA in D5 cells after cleavage with BalI and SmaI. The probe was complete virion DNA. (E) represents early passage DNA (passage 9 to 10) and (L) represents late passage DNA (passage 118) of the D5 clone.*

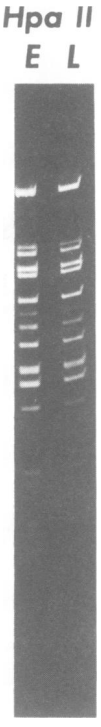

FIG. 8. *Restriction enzyme patterns of AAV-2 DNA rescued from latently infected D5 cells. DNA was extracted, as described, from AAV-2 virions that were rescued upon challenge with adenovirus type 2. After digestion with HpaII, the DNA digests were fractionated by electrophoresis in a 6% polyacrylamide gel. (E) AAV-2 DNA extracted from stock virus, and (L) DNA extracted from AAV-2 virions rescued from D5 cells.*

the AAV sequences detectable appear to be associated with cellular DNA. We base this conclusion on the facts that all of the viral sequences migrate more slowly than free AAV DNA in agarose gels and that two of the three restriction enzymes available which do not cleave AAV virion DNA reduce the apparent molecular weight of DNA-containing AAV sequences. A plasmid containing only AAV DNA would not be expected to be cleaved with these enzymes. It is important to note, however, that this does not demonstrate integration into chromosomal DNA. Plasmids containing DNAs of both viral and cellular origin are also possible.

Recombination between AAV and cellular DNAs involves the termini of AAV DNA. In experiments with *Hinc*II and *Pst*I (both enzymes with three cleavage sites in the genome), no normal terminal fragments were apparent. All terminal sequences were in fragments of higher-than-normal molecular weight with one exception (*Hinc*II no. 6, Fig. 4). On the other hand, all sequences found in internal fragments, with again a single exception, were found in fragments which corresponded to those produced by digestion of virion DNA. The single exception was *Hinc*II no. 3 which corresponded to the sum of the *Hinc*II-A and -C fragments and contained both A and C sequences. With *Pst*I digestion, all internal sequences from 0.08 to 0.92 on the genomes were associated with fragments equivalent to those produced by digestion of virion DNA. Thus, there is no evidence for association of internal AAV sequences with non-AAV DNA.

Clearly, some rearrangements or modifications of AAV DNA have occurred. Loss of the *Hinc*II-AC site in some copies and the *Hinc*II no. 6 fragment containing B sequences are examples. Additionally, *Bam*HI digestion of the integrated DNA yields a fragment with a molecular weight of 1.85×10^6 which contains sequences from both ends of the genome but which is only two thirds of unit length, thus implying a large deletion. However, there are four reasons for concluding that at least some of the integrated copies of the genome are intact. (i) With the exception of the *Hinc*II-AC fusion product, all internal sequences from 0.08–0.92 detectable after *Hinc*II or *Pst*I digestion are associated with fragments corresponding to those of virion DNA. (ii) Digestion of integrated DNA with three one-cut enzymes leads to products of unit length in

each case. (iii) Free copies of the genome appear in DNA from late but not early passage cells. These copies are indistinguishable from virion DNA. (iv) Infectious virions containing DNA indistinguishable from that produced in lytic infections can be efficiently rescued from both early and late passage cells.

At least some of the integrated copies of the genome appear to cluster in a tandem array. AAV-specific fragments produced by enzymes which do not cleave virion DNA are of high molecular weight ($>20 \times 10^6$). In fact, we cannot resolve more than a single species, although the poor resolution in this molecular weight range makes determination of the precise number of such species difficult. If the integrated copies cluster, the opposite termini of the genome should be brought into close apposition. Indeed *Hin*cII no. 2 and 4 (Fig. 4) contain sequences from both termini. Furthermore, no. 4 is precisely the size expected for a fragment containing only sequences from the two normal *Hin*cII terminal fragments, B and D. *Hin*cII no. 2 is larger than expected for a simple joining of B- and D-terminal sequences. Either the terminal sequences have been reiterated, or cellular sequences have been interposed. Further studies with two enzymes which cleave virion DNA once, *Bam*HI and *Hin*dIII, support this notion. Fragments which contain AAV sequences from both sides of the cleavage site and which are of unit length and larger were produced. The *Hin*cII-BD fragment is consistent with a head-to-tail tandem. No evidence was obtained for a head-to-head tandem. Data (not presented) which led to a similar conclusion were obtained with *Sal*I (one cut), *Eco*RI (two cuts), and *Pst*I (three cuts).

As with other eucaryotic systems involving the integration of exogenous DNA into cellular DNA, this system is complex. Another approach to the number of insertion sites is to consider the number of species containing AAV terminal sequences which may be associated with cellular sequences. However, the number detectable is variable with different restriction enzymes. The situation is further complicated by the possible insertion of relatively short segments of cellular DNA between adjacent copies of the AAV genome within a cluster. Determination of the exact nature and number of joints between viral and cellular DNAs will require cloning of specific fragments and subsequent sequencing.

The mechanism by which free copies of the genome are generated in late passage cells and by which infectious virus is rescued from both early and late passage cells by addition of helper virus is unknown. Only forms equivalent to the linear mature virion genome appear to be rescued in either case. Recombination would appear to be required in any model to free the genome from its integrated state, because there is no evidence of free DNA in early passage cells, yet these cells go on to produce late passage cells containing free viral DNA, and helper-induced rescue is equally efficient from both early and late passage cells. Such recombination might be intrastrand, interstrand (unequal crossing over) (2), or intrastrand after the "onionskin-replication-peeling out" model proposed for rescue of simian virus 40 by Botchan et al. (7). The latter model might be especially attractive to explain helper-induced rescue, because the addition of helper virus would provide the products needed for AAV DNA replication. However, it is not clear where such required components would originate during continued passage to account for the free DNA in late passage cells of the clone. Because of the nature of the free DNA (i.e., equivalency to virion DNA) and because it contains the origin of DNA replication (4, 13, 14, 25), the terminal repetition of AAV DNA would appear to be intimately involved in any of these potential mechanisms. In this light it is of particular interest that the only sequences altered with continued passage of the clone are within the palindromic region of the terminal repetition as shown by the *Sma*I results.

The nucleotide sequence orientation in AAV DNA bears a striking resemblance to that found in bacterial transposons, and the ability of AAV DNA to recombine in and out of host DNA is similar to the genetic properties of both transposons and insertion sequences. In this sense AAV may represent the eucaryotic equivalent of these genetic elements. Present data indicate that AAV latent infection may be a widespread phenomenon (e.g., 20% of AGMK cell lots). The consequences for the host are uncertain at this time. Because of the ability of AAV to inhibit the replication and oncogenicity of adenovirus (9, 19, 21) and the oncogenicity of herpes simplex virus (6), latent infection by AAV could conceivably have a protective effect.

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