

Site-specific integration by adeno-associated virus

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ABSTRACT Cellular sequences flanking integrated copies of the adeno-associated virus (AAV) genome were isolated from a latently infected clonal human cell line and used to probe genomic blots derived from an additional 21 independently derived clones of human cells latently infected with AAV. In genomic blots of uninfected human cell lines and of primary human tissue, each flanking-sequence probe hybridized to unique bands, but in 15 of the 22 latently infected clones the flanking sequences hybridized not only to the original fragments but also to a total of 36 additional species. AAV probes also hybridized to 22 of these new bands, representing 11 of the 15 positive clones, but never to the fragment characteristic of uninfected cell DNA. From these data we conclude that the AAV genome preferentially integrates into a specific region of the cellular genome. We have determined that the integration site is unique to chromosome 19 by somatic cell hybrid mapping, and this sequence has been isolated from uninfected human DNA.

Latent infection by the human dependovirus adeno-associated virus (AAV) was discovered by Hoggan and collaborators (1) during the screening for cryptic infection of primary African green monkey kidney cells and human embryonic kidney cells intended for vaccine production. Although all cell lots were initially negative for AAV antigen, challenge by infection with adenovirus led to positive AAV responses in up to 20% of the monkey cell lots and in 1–2% of the human cell lots. Thus, AAV latent infection appeared to be a rather frequent natural occurrence.

Under physiological conditions AAV can replicate in cell culture only in the presence of a coinfection by a helper virus, either an adeno- or a herpesvirus (2). In the absence of helper virus, the AAV particle can penetrate to the cell nucleus, where the linear single-stranded DNA genome is uncoated, although no virus-specific macromolecular synthesis is detected (3). Under these conditions the viral DNA can then integrate into the cellular genome to establish a latent infection from which the integrated viral genome can be activated and rescued by superinfection with helper virus (1).

Latently infected cells were produced *in vitro* by infection with AAV at high multiplicity (250 infectious units per cell) in the absence of helper virus coinfection (1, 4). Initial studies to characterize the state of viral DNA in latently infected cells were done by reassociation of denatured genomic DNA in solution (5) and by Southern blots of genomic DNA digested with restriction endonucleases that do not have a recognition site within the AAV genome (6). The proviral DNA was found to be covalently linked to high molecular weight cellular DNA (5, 6), and in rescuable clones several copies of the viral genome were present in tandem arrays (6–8). The

viral DNA contained palindromic inverted terminal repeats that appeared to be at or near the junctions with the cellular sequences.

Further characterization of the proviral sequences was done by digestions of genomic DNA from latently infected cells with a series of restriction endonucleases. Hybridization with AAV DNA-specific probes produced a distinct pattern of fragments for every clone examined. Because the sizes of the putative viral–cellular junction fragments were different in every clone, it was concluded that the viral DNA integrated into random sites within the cellular genome (6–8).

Recently, Kotin and Berns (9) reported on the molecular cloning of integrated AAV sequences from a clone of latently infected human Detroit 6 cells, clone 7374. Two of the molecular clones isolated contained viral–cellular junctions, which were sequenced. The two flanking cellular sequences were hybridized to genomic blots of uninfected cell DNA and it was found that both flanking sequences were present at most only once or a few times in the human genome (e.g., each hybridized to only a single and different *Bam*HI fragment). In this paper we report on the use of these flanking sequences as probes of genomic blots of 21 additional, independently derived clones of latently infected human cells obtained from three more laboratories. All but two of the cell lines that were screened contained proviral DNA that was rescuable upon superinfection with adenovirus. In at least 15 of the clones, there was evidence that at least one copy of the original sequence had been altered in size as a result of viral DNA integration. From these results it appears that in a majority of these clones (15 out of 22), the AAV genome integrated into a specific site, which we have mapped to chromosome 19. To our knowledge this is the first instance in which site-specific integration by a mammalian DNA virus has been demonstrated.

METHODS

Probes. DNA flanking the provirus of Detroit 6 cell line 7374 was obtained and used as probes (9). The flanking cellular sequences were designated “left” or “right” with respect to the viral sequence. Left and right flanking probes were produced as described (9) (see Fig. 1).

AAV probe was generated from wild-type virion DNA (10). AAV-neo probe was produced from cloned AAV DNA in which the open reading frame encoding the capsid gene was replaced with the gene for neomycin resistance (11). Probe was prepared by random oligonucleotide priming (12).

Genomic DNA Analysis. High molecular weight DNA was extracted from cells essentially as described by Maniatis *et al.* (13). The DNA (10–15 μ g) was digested to completion with an excess of the appropriate restriction endonuclease under

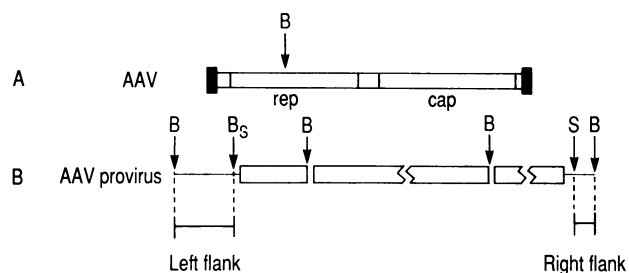


FIG. 1. AAV genome and provirus. (A) The viral genome with the unique *Bam*HI site is shown as an open box with the terminal repeats represented by filled boxes. The positions of the two viral open reading frames (ORFs), designated rep and cap, are indicated. The rep ORF encodes functions necessary for control of viral replication and gene expression. The cap ORF encodes the viral structural proteins. (B) Organization of proviral and cellular DNA from a Detroit 6 cell line, 7374. The single line represents cellular sequences. Probes were derived from *Bam*HI-*Bst*EII and *Sst* I-*Bam*HI subfragments, which correspond to the left and right flanks, respectively. B, *Bam*HI; B_s, *Bst*EII; S, *Sst* I.

conditions recommended by the vendor. The DNA digests were fractionated by agarose gel electrophoresis in TBE buffer (90 mM Tris borate/2 mM EDTA, pH 8) and capillary-blotted onto nylon membranes (14). The filters were prehybridized 2 hr and hybridized 18 hr at 66°C. The filters were washed once with 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS/25 mM sodium phosphate, pH 7.4, and twice with the same buffer containing 0.2× SSC at 66°C. The hybridized probe was detected by autoradiography using Kodak XAR film. Two Lightning Plus (DuPont) intensifying screens were used for the genomic blots.

λ Library. A commercially prepared λ genomic library produced from human embryonic fibroblasts (cell line WI-38)

(Stratagene) was initially screened using *Escherichia coli* strain P2392 (Stratagene), which selects for λ recombinants. Subsequent screenings were done using *E. coli* strain LE392 (13). Plaques were transferred onto duplicate filters (13) and hybridized to left and right flanking probes. Positive plaques for both left and right were picked and the eluted phage were replated at lower density and screened with either left or right flanking probes. Positive plaques were picked and the phage were grown in liquid cultures of *E. coli*. The phage from the lysed bacteria were concentrated and the DNA was extracted (13).

Cell Lines. Latently infected KB cell lines M19, M21, M26, M32, M50, M53, M69, M77, and M104 were cloned as described (7). Latently infected HeLa cell lines G11, H3, C11, and F10 were produced essentially as described (4). A description of the proviral organization of these HeLa cell lines will be published elsewhere (R.J.S., X.Z., and L.H.). Latently infected Detroit 6 cell lines S105, S107, S109, S110, S111, S115, S119, and HN21 were produced by selection in medium containing the neomycin analogue G418, as described (8). Cloning of the latently infected Detroit 6 cell line 7374 has been described (4).

Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum plus penicillin and streptomycin (100 units/ml and 0.1 mg/ml, respectively).

Somatic Cell Hybrid Panels. Three panels of somatic cell hybrid DNA were used to localize the integration site to a single chromosome. The first two panels were kindly provided by K.-H. Grzeschick (University of Marburg, F.R.G.; ref. 15). A third somatic cell hybrid panel was constructed and analyzed as described (16).

RESULTS

Kotin and Berns (9) found that the flanking-sequence probes, arbitrarily designated left and right, each hybridized to discrete *Bam*HI fragments [3.6 kilobases (kb) and 2.6 kb,

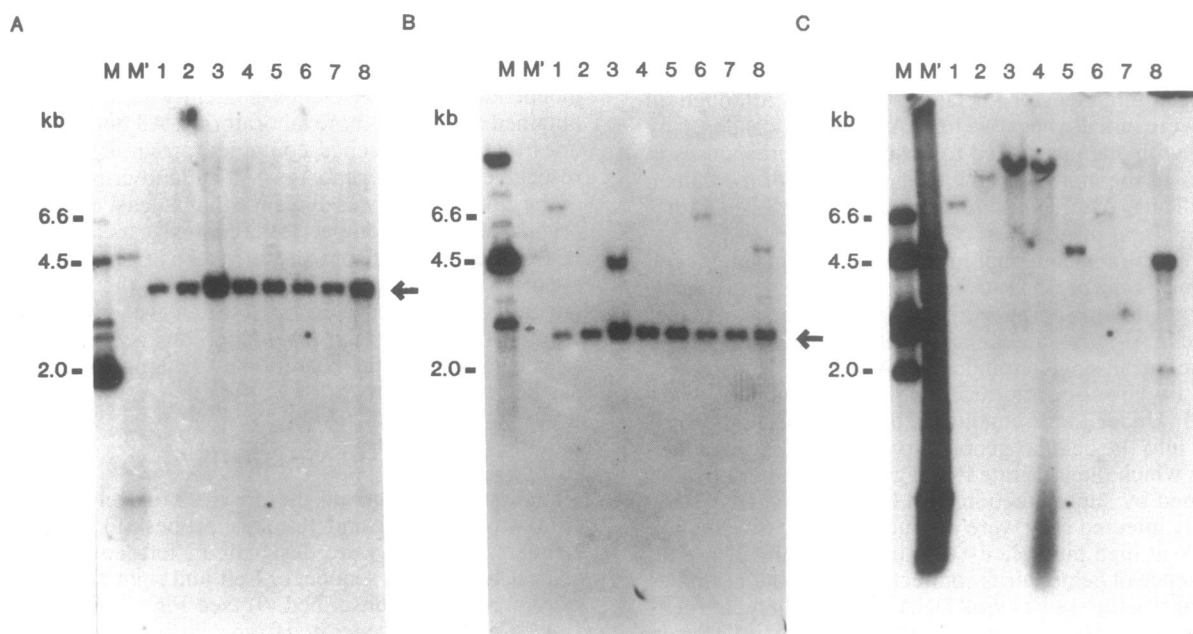


FIG. 2. Analysis of genomic DNA from latently infected Detroit 6 cells. Cellular DNA ($\approx 10 \mu\text{g}$ per lane) was digested with *Bam*HI and fractionated by electrophoresis in a 0.7% agarose/TBE gel. The DNA was transferred onto a nylon membrane and initially hybridized to right flank probe (B). After autoradiography, the membrane was stripped of probe in 0.2 M NaOH/5 mM EDTA. The filter was then hybridized to an AAV/neomycin gene-specific probe (C). After autoradiography, the membrane was stripped of probe and hybridized to left flank probe (A). Lanes: 1, cell line S105; 2, S107; 3, S109; 4, S110; 5, S111; 6, S115; 7, S119; 8, HN21. For lane M, three proviral isolates digested with *Bam*HI ($\approx 1 \text{ ng}$ of each) and $\approx 500 \text{ ng}$ of λ DNA digested with *Hind*III (for use as UV fluorescent size markers) were combined. For lane M', cloned AAV DNA digested with *Pst* I was combined with intact virion DNA. In A and B, arrow indicates the band common to both latently infected and uninfected cellular DNA. The 23-kb, 9.4-kb, and 6.6-kb bands in B resulted from hybridization to the λ DNA markers. The level of hybridization is not significant, since there was $\approx 10^5$ times the molar amount of the λ DNA as compared to the single-copy sequence detected in the genomic DNA. The extent of carryover of signal after probe stripping can be determined by comparing lanes M' in A and C.

respectively, which are referred to as the original bands or sequences] in genomic blots of uninfected cellular DNA. In genomic blots of the latently infected clone from which the flanking sequences had been isolated, Detroit 6 clone 7374, these probes hybridized to the same bands as in the uninfected cellular DNA as well as to several new bands. Thus, it appeared as though one copy of the original sequence had been disrupted and one (or more) had remained intact. Significantly, the new bands also hybridized to AAV-specific probes but the original band did not. Furthermore, the flanking cellular probes were found not to have sequence similarity to AAV DNA as determined by sequence analysis (ref. 9; R.M.K. and K.I.B., unpublished observations).

Previous characterizations of independently derived clones of cells latently infected with AAV were based on restriction digestion and blotting of genomic DNA. The putative junction fragments were distinct in each of the cell lines and as a consequence it was assumed that AAV DNA integrated at random sites in the genome. The results of Kotin and Berns (9) indicated that extensive sequence rearrangement was associated with viral DNA integration affecting both the viral and cellular sequences, making the original conclusion less certain. The isolation of the cellular flanking sequences enabled us to assess directly whether integration of AAV DNA consistently disrupts the same cellular sequence.

A total of 22 independently derived clones of human cells latently infected by AAV were screened with cellular sequences derived from the viral-cellular junctions of the Detroit 6 clone 7374 (Fig. 1 and ref. 9). All the latently infected cell lines tested showed the two original bands detected by the left and right flanking probes common to uninfected cells (arrows in Fig. 2 A and B), and 15 of these cell lines showed new bands in addition to the original bands (Fig. 2; Table 1). The 22 clones were independently derived in four different laboratories and uninfected Detroit 6, HeLa, and KB cells were used in different instances. Using the left and right flanking-sequence probes, we have observed only the two original *Bam*HI fragments in any of the uninfected cell DNAs. Only the same two bands were observed in genomic blots of DNA from human placental and fetal tissue (data not shown). Genomic DNA from 13 sister clones of Detroit 6 cells that either were negative for AAV or contained <10% of the AAV genome by design produced only a single band when hybridized to the right flank probe. Ten clones of mouse-human hybrid cells containing human chromosome 19 also showed only the two bands characteristic of uninfected cells. Thus, to date the polymorphism has only been observed after exposure to wild-type AAV virus.

None of the original uninfected cells contained detectable AAV sequences**. In the 15 positive clones there are 36 new bands detected with the left and right flank probes. In 11 of the 15 positive clones, AAV-specific probe hybridized to 22 of these additional fragments. Seven of the 36 fragments hybridized to left flank probe, and AAV DNA was associated with 4 of the 7 fragments. The right flank probe also hybridized to 3 of the 7 left-flank-positive fragments, which is indicative of cellular sequence rearrangement. The right flank probe hybridized to 29 new bands, 18 of which were positive to AAV probe. For 13 of the 21 clones, genomic hybridizations were performed on parallel blots of identically digested

DNA as well as by rehybridization of stripped blots. By either procedure the results were the same. In every cell line in which the original left flanking sequence was disrupted, the original right flanking sequence was disrupted as well, but the converse was not the case.

Seven of the clones tested were created by infection with viral constructs from which both AAV major open reading frames had been deleted and replaced by the heterologous gene for neomycin resistance (8) (Fig. 2; Table 1). Three of the seven clones of this type also showed disruption of the original sequence homologous to the right flanking-sequence probe. These data suggest the possibility that none of the known AAV gene products is required for site-specific integration. A caveat is that the viral vector preparation was not totally free of wild-type AAV; thus the specificity of integration may have been a consequence of complementation with wild-type virus.

Table 1. Summary of hybridization of three probes to *Bam*HI-digested DNA from latently infected cells

Cell line	No. of supernumerary bands					
	R	L	Both	AAV + R	AAV + L	AAV + both
Detroit 6						
7374	2	1	0	2	1	0
S105*	1	0	—	1	0	—
S107*	0	0	—	0	0	—
S109*	1	0	—	0	0	—
S110*	0	0	—	0	0	—
S111*	0	0	—	0	0	—
S115*	2	0	—	2	0	—
S119*	0	0	—	0	0	—
HN21*	2	0	—	0	0	—
HeLa						
G11	4 (+1?) [†]	4	1	(4 [‡])	(3 [‡])	1 [‡]
H3-1	3 (+1?) [†]	1	1 [‡]	1	1 [‡]	1 [‡]
C11	3 (+1?) [†]	0	—	1	0	—
F10	0	ND	—	0	ND	—
KB						
M19	2	0	—	2	0	—
M21	3 (+1?) [†]	0	—	3	0	—
M26	1 (+1?) [†]	0	—	1	0	—
M32	1	1	1	1	1	1
M50	0	0	—	0	0	—
M53	0	0	—	0	0	—
M69	1	0	—	0	0	—
M77	2	0	—	0	0	—
M104	1	0	—	1	0	—
Total bands	29	7	3	18	5	3
Positive clones, no. (%)	15 (68)	4 (18)		11	3	

The supernumerary bands are listed for the right (R) and left (L) flank probes. The number of supernumerary fragments associated with AAV DNA as determined by hybridization to viral-specific probe is indicated under AAV + R or AAV + L. Parental, uninfected cellular DNA produced a single band when hybridized to either left or right flank probe. Fragments that hybridized to left and right flank probes or to left flank, right flank, and AAV probes are listed under Both and AAV + both, respectively. No signal was detected when uninfected cellular DNA was hybridized to AAV-specific probe (except for one KB cell line; see text footnote**). ND, not determined.

*Cell line infected with an AAV vector containing the neomycin-resistance gene.

[†]The presence of one additional band is uncertain.

[‡]It is uncertain whether there is an overlap of the fragments detected by the probes. This was the result of comparisons between parallel blots hybridized to different probes as opposed to blots that were hybridized, stripped, and then rehybridized to the other probe.

**The current KB cell control supplied from the Laughlin laboratory, however, did contain AAV-specific bands but did not have additional bands that hybridized to the flanking-sequence probes. The pattern of AAV fragments seen in the genomic blots of the so-called uninfected KB cells differed from all other latently infected clones. In the original report (7), the KB cells were negative for AAV sequences prior to AAV infection; therefore, the parental KB cell line was apparently infected with AAV subsequent to the establishment of the originally reported latently infected cell lines.

To determine the chromosomal assignment of the viral integration, the left and right flanking sequences were used as probes on two panels of *EcoRI*-digested DNAs from rodent-human somatic cell hybrids. The analysis of the concordance between the retention or loss of a specific chromosome and the presence or loss of the autoradiographic signal with these probes localized the viral integration site to chromosome 19. To unequivocally make the chromosomal assignment, the same probes were hybridized to a panel of 18 rodent-human hybrid DNAs. This set of hybrids included 4 that had retained autosome 19 in 100% of the cells examined and 14 that did not retain autosome 19. The results of these studies are summarized in Table 2, where it is apparent that human autosome 19 is the only chromosome whose retention/loss correlates unequivocally with the presence/absence of the signal for homology elicited by both probes.

Bacteriophage λ libraries produced from the human embryonic fibroblast cell line WI-38 were screened with probes derived from the cellular DNA flanking the provirus in the latently infected Detroit 6 cells (4, 9). The DNA from the recombinant phage that hybridized to both left and right flank probes was analyzed by restriction mapping, Southern hybridization, and limited sequencing. The left and right flank probes hybridized to a single 7.6-kb *EcoRI* fragment that was the same size as the fragment produced by *EcoRI* digestion of genomic DNA, demonstrating that both left and right flank probes were derived from a small region of the genome. Digestion with *BamHI* produced a fragment specific to the left flank probe that was the same apparent size as seen in a *BamHI* genomic digest. However, fragment that was specific to the right flank probe was not the same size as generated by *BamHI* digestion of genomic DNA (data not shown). These

results indicate that the original, unoccupied sequence was altered in some way by propagation in the λ library or by subsequent cloning procedures. Preliminary sequencing results showed that the right flank probe sequence was colinear with the corresponding region of the uninfected cellular DNA (data not shown). Hybridization to AAV probe showed no evidence of the presence of viral DNA in the phage insert (data not shown).

DISCUSSION

In this paper we have presented evidence that in a high percentage of cases, the AAV2 genome is integrated at a specific site on chromosome 19 to establish a latent infection in human cells. All mammalian nuclear DNA viruses can establish persistent infections of the intact host, often in a latent form in which virus-specific macromolecular synthesis is difficult to detect. The genome may persist either as an extrachromosomal element or as a provirus integrated into the cellular genome (e.g., human papilloma virus and AAV, respectively). In either state replication of the viral genome is tightly synchronized with that of the host. The only known occurrences of site-specific integration have involved retroviruses that induce specific cancers and have been found to insert adjacent to cellular oncogenes [e.g., the avian leukosis viruses (17)]. However, the specificity is related to selection for tumor formation, and insertion of the avian leukosis virus genome is most often at other sites in the genome. Under nonselective conditions, Rous sarcoma virus DNA was found to integrate into a large, albeit finite, number of sites (18), whereas AAV DNA integrated at a specific locus in 68% of the cell lines examined. It remains possible that AAV DNA

Table 2. Somatic cell hybrid analysis

	Human chromosomes retained																						X
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
Positive clones																							
HY.22AZA1	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	+	+	-	-	-	t ₁
HY.36.1	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+
YC2T1	+	-	-	-	-	-	-	-	-	-	+	+	-	*	-	-	-	+	+	+	-	-	+
Y.XY.8F6	-	-	+	+	+	+	+	-	-	+	+	+	-	-	-	+	+	-	+	-	-	+	+
LB250 (human DNA)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Negative clones																							
HY.112F7	-	-	+	+	-	-	-	+	-	*	-	-	-	-	-	-	-	-	-	+	-	-	q
HY.19.16T3D	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	+	-	+	-	-	q-
HY.31.24E	-	-	-	-	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+
HY.60A	-	-	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	+
HY.70B1A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	t ₂	-	t ₂
HY.70B2	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	-	-	-	-	t ₂	-	t ₂
HY.75E1	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+
HY.94A	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
HY.94BT1	-	-	-	+	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-	t ₃
HY.95A1	-	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+
HY.95B	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+
HY.95S	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
RJ.369.1T2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
Y.173.5CT3	+	-	+	+	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	i
No. concordant																							
+/+	1	0	1	1	2	1	1	1	0	1	3	3	0	2	0	1	2	2	4	1	0	1	4
-/-	13	13	10	10	10	8	11	9	12	11	10	9	9	8	10	12	13	10	14	10	9	10	1
No. discordant																							
-/+	1	1	4	4	4	6	3	5	2	3	4	5	5	6	4	2	1	4	0	4	5	4	13
+/-	3	4	3	3	2	3	3	3	4	3	1	1	4	2	4	3	2	2	0	3	4	3	0
% discordancy	22	28	39	39	33	50	33	44	33	33	28	33	50	44	44	28	17	33	0	39	50	39	72

Positive clones are those hybrids which gave signal when hybridized to probes derived from the cellular sequences flanking the provirus. Negative clones produced no autoradiographic signal. Concordance indicates the presence or absence of signal with that human chromosome, +/+ and -/-, respectively. Discordancy was determined by retention of a particular chromosome and loss of a signal or loss of a particular chromosome but not the signal. *, less than 100% of the chromosome was present in the metaphase spreads examined; i, isochromosome; t, translocation; t₁, t(X;X); t₂, t(X;21); t₃, t(X;Y); q, loss of p arm; q-, loss of q arm.

also integrates at sites where rescue cannot occur, but since in all but two cell lines examined in this study rescue did occur it is possible that rescue itself is a selectable phenotype.

The apparently random integration by most DNA viruses of higher eukaryotes is similar to that of bacteriophage Mu and contrasts to the site specificity of integration by the lambdoid bacteriophages. The case of the AAV2 integration is unique in that a specific site within the human genome is specifically recognized at a high frequency in established cell lines. Whether the recognition is directly at the level of DNA sequence is not known. We have isolated the unoccupied site from uninfected cells, and the original right junction site has been identified by limited sequence analysis. In this region we see stretches of homology only to the extent of 6 or 7 bases between the viral and cellular DNA, which could correspond to the "patchy" homology observed in other systems at junctions between viral and cellular DNA. Thus, integration does not seem to be the consequence of homologous recombination, although the specificity may still be at the nucleotide level. This may be the case with adenovirus DNA integration, which appears to occur at preferred sites. A recent paper has demonstrated that adenoviral DNA fragments can recombine with cloned pre-integration sites in a cell-free extract, whereas recombination between adenovirus DNA and random sequence was not observed in the *in vitro* system (19). On the other hand, the target site for AAV DNA integration may result from a higher order of structure at the chromatin level. Preferential integration of DNA viruses at specific cytogenetic sites associated with constitutive chromosomal fragility has been reported (20–22). Integration of adenovirus–simian virus 40 hybrid DNA at the highly recombinogenic site at chromosome 1p36 has been observed (23). However, specificity of integration was at the chromosomal level and not at the molecular level.

An alternative explanation of our results is that rather than being 22 independently derived clones, all of the clones investigated were the progeny of a single original latently infected cell. We reject this possibility for several reasons. (i) Not all of the clones showed disruption of the common cellular integration site. (ii) Where disruption was seen the new fragments were of different mobilities in every case. (iii) The integrated AAV sequences produced different patterns in every case, even though in the one instance where one of the clones had been followed for >100 passages, the pattern of *Bam*HI fragments did not change. (iv) All of the clones obtained from the Muzyczka laboratory were latently infected by vectors containing the neomycin-resistance gene. Thus, every clone positive for disruption of the common site shows restriction polymorphisms different from every other positive cell and from the parental cells.

Experiments in collaboration with J. Menninger and D. Ward have directly demonstrated that the unoccupied site is specific to chromosome 19q (data not shown).

The specificity of AAV integration impinges on its use as a vector and the consequent opportunity to study the regulation of human genes at a specific site in the genome.

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