Adeno-associated Virus Type 2: A Latent Life Cycle[1]

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Adeno-associated viruses (AAV) constitute the *Dependovirus* genus of the family Parvoviridae, which are small DNA viruses with a host range extending from insects to humans (*1*). Those with vertebrate hosts belong to the subfamily Parvovirinae, whose members include both viruses with life cycles that are predominantly, if not completely, characterized by productive infection, and viruses that establish latent infections as a prominent compo-

[1] Abbreviations: AAV, adeno-associated virus; VP, viral protein (capsid); nt, nucleotide; *orf*, open reading frame; NTP, nucleoside triphosphate; CAT, chloramphenicol acetyltransferase; *itr*, inverted terminal repeat; SV40 *ori*, simian virus 40 origin of DNA replication; RFLP, restriction-fragment-length polymorphism; kb, kilobase; EBV, Epstein–Barr virus; EBNA1, EBV nuclear antigen 1; MOI, multiplicity of infection; E1b, adenovirus early reg. 1B; CFTR, cystic fibrosis transmembrane conductance regulator; *aavS1*, cellular genetic locus of AAV integration.

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nent of their life cycles. The AAV are members of the latter group; as such, they do not normally undergo a productive infection in cell culture unless there is coinfection with a helper virus, either an adeno- or a herpesvirus (*2–5*). Because of the requirement for a helper virus coinfection in cell culture, AAV was initially considered to be defective. We now recognize that the requirement for helper virus coinfection reflects a tightly regulated life cycle, oriented to the latent infection of a healthy host cell. AAV is widespread in the human population; about 90% of U.S. adults are seropositive (*6–11*). The virus has never been causally associated with any disease; indeed, it has demonstrated anti-oncogenic properties in model systems (*12–20*).

I. Structure

The Parvovirinae are among the smallest of the DNA animal viruses, possessing a naked icosahedral capsid of 20–26 nm in diameter (*3*). The crystal structures of several parvoviruses closely related to AAV have been determined (*21–24*). An interesting feature of the interior capsid is the existence of 60 pockets, each capable of accommodating, and thus immobilizing, 11 bases of the genome. There are three structural proteins with overlapping amino-acid sequences that differ in size by extension of the NH_2-terminus (*25–27*). The predominant AAV coat protein is the smallest, VP3. The extended NH_2-terminus of the largest coat protein, VP1 ($\approx$10% of total protein mass in the crystal structure), extends into the interior of the capsid and is thought to interact with the genome. The virion is resistant to heat (56°C, 30 minutes), lipid solvents, and acid, but is highly susceptible to alkaline conditions (pH >11.0) (*4, 28*).

The genome is a linear single-stranded DNA of about 4.7 kb. AAV is unusual for a single-stranded DNA virus in that strands of both polarities are separately encapsidated in different virions with equal frequency (*29*). When the virion DNA is extracted, the complementary strands anneal to form duplex molecules, unless special precautions are taken to prevent hybridization (*29, 30*). This curious state of affairs was initially demonstrated by isolation of single-stranded DNA under conditions that prevented annealing, but that did not denature DNA, and by showing that the complementary strands in the duplex virion DNA molecule originated from different virions (*30, 31*).

Finally, virions containing DNAs of differing polarities could be physically separated (*32*). There is an inverted terminal repeat at the ends of the DNA that contains a symmetrical sequence of 125 bases (*33*); consequently, the ends of the single-stranded genome can either base-pair with each other to form a hydrogen-bond-stabilized single-stranded circle, or fold on themselves to form duplex regions at the ends of linear single-polynucleotide chains (*34, 35*).

II. Genetic Map

The genome of AAV2, the most common human AAV, has been sequenced (*36*). As stated above, there is an inverted terminal repeat of 145 bases, of which the terminal 125 bases are symmetrical. There are smaller internal palindromes on either side of the overall axis of symmetry, so that, when the terminal symmetrical sequence is folded on itself to maximize base-pairing, a T-shaped structure is formed (see Fig. 1). Of the 125 bases in the symmetrical sequence, only seven are unpaired: three where each of the small internal palindromes fold back on themselves, and a single base between the two internal palindromes at the axis of symmetry. Interestingly, all of the unpaired bases are As or Ts and all of the base-pairs in the cross-arms of the T are G·C base-pairs, with one exception.

There are two sites of heterogeneity at the ends of the genome. The first is at the very ends of the genome where one or both of the 5′-terminal Ts or 3′-terminal As may be missing (*37*). The heterogeneity appears to reflect slight variability at the site of a nicking reaction that occurs during DNA replication. The second heterogenous sequence occurs in the small internal palindromes [from nucleotides (nts) 42 to 84] and is the consequence of inversion of the terminal palindromic sequence during DNA replication (*38–40*).

There are two large open reading frames (*orf*s) of roughly equal size in the internal unique sequence of the genome (see Fig. 2). Detailed genetic studies have helped to elucidate the functions of the proteins encoded. The studies have been enhanced by the infectivity of the cloned duplex form of the AAV genome (*40–42*). Transfection into human cells infected by helper adenovirus leads to rescue from the plasmid vector and replication of the AAV genome. Thus, any mutant can be propagated in *Escherichia coli*, and the phenotype in human cells can be assessed directly.

The *orf* in the left half of the genome has been called the *rep* gene because almost all mutations in this region block DNA replication (*43–45*). There are promoters at map positions (mps) 5 and 19 for transcripts that cover the *orf* and extend to a polyadenlyation signal at mp 96 (*36, 46, 47*). There is an intron near the 3′ end of the *orf* (nts 1907–2227), so that both spliced and unspliced forms of both transcripts are found in the cytoplasm and are translated (*48, 49*). Thus, there are four *rep* gene products that play major regulatory roles during AAV infection. The two larger proteins, Rep68 and Rep78, can bind specifically to the AAV terminal repeat (*50*) and can function as both site-specific nickases and DNA helicases during replication (*51–53*). All of the *rep* proteins contain an NTP binding site homologous to NTP binding sites of the T antigens of polyomaviruses and the E1 proteins of papillomaviruses (*54–56*).

The right *orf* encodes three structural (viral) proteins, VP1–VP3. The

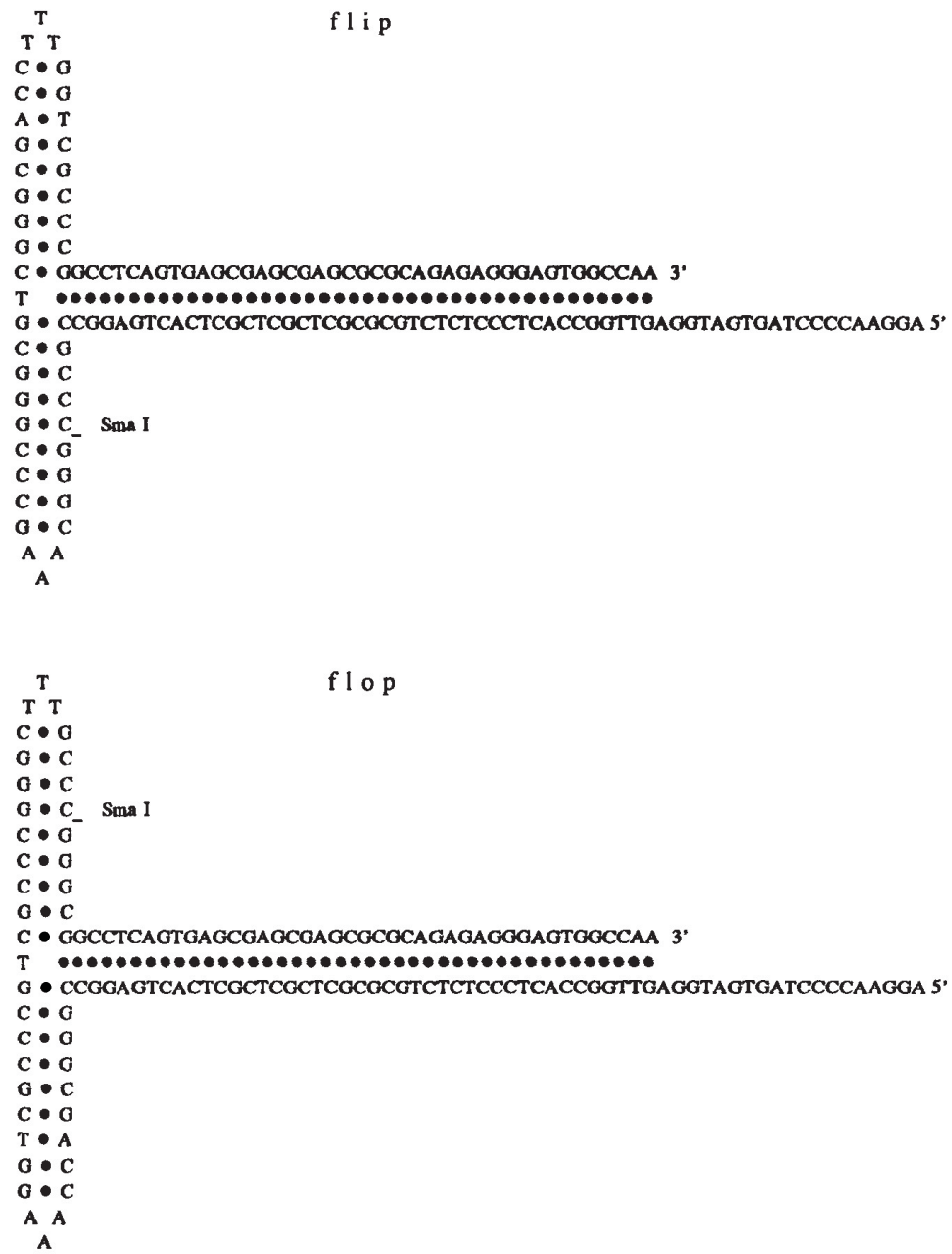


FIG. 1. The AAV terminal repeat is extensively self-complementary and can form a stable hairpin structure. Of the 125 palindromic bases, only seven are not base-paired. The two isomeric forms of the AAV *itr* were arbitrarily denoted "flip" and "flop." The restriction enzyme *Sma*I cuts only one of the two smaller internal palindromes.

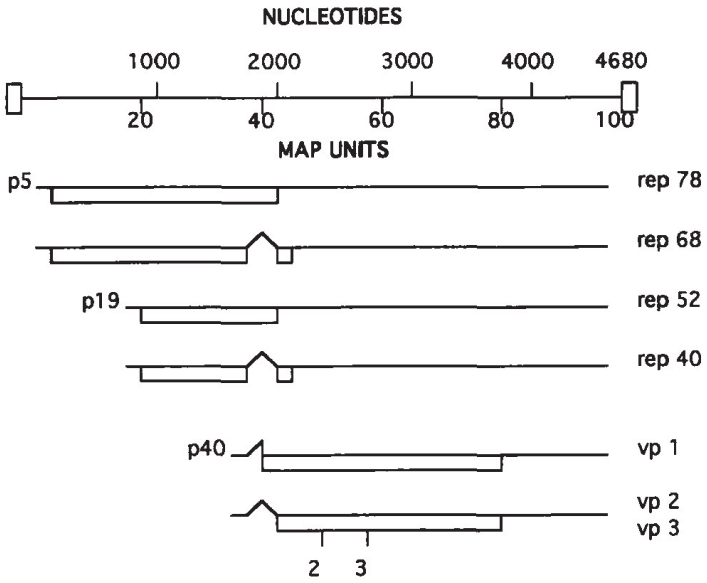


FIG. 2. The promoter at map unit 5 (p5) initiates a transcript that is either translated intact (encoding Rep78) or spliced to encode Rep68. The promoter at map unit 19 (p19) initiates a transcript that is either translated intact (in-frame with the larger *rep* proteins), yielding Rep52, or spliced to yield Rep40. The promoter at map unit 40 (p40) initiates all three capsid mRNA species. VP1 is produced by an alternative splicing event, VP2 and VP3 are produced by the same splice sites used for the *rep* proteins, VP2 results from the utilization of an alternative ACG start codon.

smallest, VP3, is the major coat protein. All three proteins are translated in the same phase from a spliced transcript originating from a promoter at mp 40 (57). VP3 translation initiates from the first AUG, which is 557 nts from the 5′ end of the most common spliced transcript. VP2 translation occurs from the same spliced transcript at an ACG upstream of the VP3 start site (58). VP1 is translated from an alternatively spliced version of the same transcript (59–60).

III. Establishment of Latency

Infection of continuous lines of human cells in culture with AAV leads to the establishment of a latent infection. AAV latency was discovered in primary cultures of human and African green monkey kidney cells (62). Although the cultures were negative for AAV antigens, "challenge" infection with adenovirus led to a productive AAV infection without addition of exogenous AAV. The same group soon discovered that, unlike other nuclear DNA vi-

ruses, AAV latent infection could be established readily in cell culture. Cultures of human cells infected at a high multiplicity of infection (MOI) (e.g., 250 infectious units per cell) and passaged blindly continued to produce AAV upon challenge with adenovirus for over 50 passages (*63*). Latently infected cells cloned at the 44th passage remained positive for over 150 passages. Thus, AAV provided a model system for the study of a latent viral infection at the molecular level. We now know that, in cell culture, AAV establishes a latent infection by integration into the cellular genome (*63–65*). In continuous lines of human cells, the integration is at a specific site in the q arm of chromosome 19 in most cases (*66–71*).

A. Negative Regulation of AAV Gene Expression

There is evidence for negative auto-regulation of AAV gene expression that leads to a latent state rather than a productive infection. The key to the regulation lies with the AAV *rep* proteins, presumably Rep68 and Rep78. Upstream of the p5 promoter is a target site for the cellular inhibitor of transcription, YY1 (*72*). In transfection experiments in which the bacterial chloramphenicol acetyltransferase (CAT) gene was driven by the AAV p5 promoter, inclusion of the YY1 binding site inhibited CAT expression; the inhibition was reversed by a concomitant adenovirus infection. Thus, it appears that the cellular milieu per se is nonpermissive for AAV gene expression.

However, the physiological significance of the result has been called into question because a second construct in which the AAV promoter sequence was extended upstream by another 16 nts expressed CAT very well (*73*). However, in a cotransfection with a construct that expressed *rep*, CAT expression was inhibited. Again, inhibition was reversed by concomitant adenovirus infection.

Since the latter experiment may be considered more physiological, it seems likely that a small amount of initial *rep* gene product negatively regulates further AAV gene expression in the absence of a "permissive" cellular milieu. Infection with a helper virus alters the milieu in a way that leads to a productive AAV infection. Although it is difficult chemically or immunologically to detect *rep* expression in nonpermissive conditions, biological effects of the expression have been observed in a number of experiments (*74–77*). In almost every instance, the effect observed has been inhibitory, and several genes, both viral and cellular, have been affected.

B. Auto-inhibition of DNA Replication

There is indirect evidence that AAV also inhibits its own DNA replication (auto-inhibition) under nonpermissive conditions (*78, 79*). The negative regulation of DNA replication was originally seen with a plasmid construct of

AAV in which the AAV p5 promoter and sequences upstream to the boundary of the inverted terminal repeat (*itr*) had been deleted and substituted by the SV40 regulatory region (nts 5240–5273-270), which contains the SV40 origin of DNA replication (*ori*). In practice, a plasmid with the SV40 *ori* will be replicated in monkey COS-7 cells which have been transformed by SV40 and constitutively express SV40 T-antigen. However, the construct containing the AAV/SV40 chimera was very poorly replicated. It was possible to restore replication either by making a frame-shift mutation in the AAV *rep* gene or by deletion of both AAV *itr*'s. Cotransfection of the two mutant constructs inhibited replication of the mutant *rep* construct.

From these results, we concluded that the *rep* product acted in *trans* to inhibit replication, and that the target sequence (either directly or indirectly) was at least partly in the *itr*. Only one copy of the *itr* was required to observe inhibition, and the relative positions of the *itr* and SV40 *ori* did not affect the inhibition. However, the effect was in *cis* (with respect to the *itr* and SV40 *ori*) and required *rep* to occur. The original plasmid could be made to undergo AAV-type replication if the COS-7 cells were infected with adenovirus; that is, the AAV DNA insert was rescued and replicated (*80*).

From these experiments, we inferred that a low level of initial *rep* expression after infection of a "nonpermissive" cell inhibits any extensive AAV DNA replication, in addition to the negative effect on AAV gene expression. A further extrapolation was that inhibition of DNA replication enhances integration of the DNA. This was based on the following experiments. A plasmid in which the bacterial gene for neomycin resistance (*neo*) was under control of the SV40 regulatory region (driven by the early promoter) replicates in COS-7 cells but does not produce stable clones of neomycin-resistant cells. On the other hand, a chimeric construct [in which the same SV40/*neo* sequences are substituted into a deletion in the structural protein *orf* (*cap* gene) of AAV] does not replicate but does integrate into the COS-7 cell genome and efficiently transforms the cells to stable resistance to G418 (M. Labow and K. Berns, unpublished). Thus, the current model is that initial *rep* gene expression after infection of a "nonpermissive" enhances establishment of the latent state by inhibiting both gene expression and viral DNA replication, which enhance integration of the viral genome.

IV. Integration

Early studies of the state of the viral DNA indicated that the DNA is integrated into large cellular DNAs (*63–81*). In the simplest cases, digestion with enzymes that do not cut viral sequences reduced the size of fragments hybridizing to viral probes on Southern blots. Thus, it was clear that the AAV

DNA had recombined with cellular sequences, but whether the DNA was actually chromosomal or in large episomal elements could only be surmised.

In the early studies one clone was followed for over 100 passages (65). Interestingly, the fragments produced by restriction enzymes that cut in the unique internal viral sequences remained unchanged; however, the pattern of fragments produced by cutting with *Sma*I, which only cuts within the *itr*, varied significantly with increased passage. It seemed that the *itr* sequences were active or changing with continued passage. This notion correlated well with the observation that all copies of the AAV DNA were integrated into large DNAs in early passages, but that a detectable fraction of the DNA was extrachromosomal after more than 100 passages. The free DNA appeared to be of unit length and duplex, and to have the same sequence orientation as virion DNA. From this we concluded that the molecular state of the viral genome in latency is both stable and dynamic during a continual latent infection.

A. Site-Specific Integration

Another issue addressed in the earlier experiments was whether integration occurs at a specific site. The rationale of the experiments was that restriction fragments representing the junctions between viral and cellular sequences would be the same when identified from different cell clones independently derived, if the integration site was constant. However, the genomic fragments identified as containing the virus–cell junctions were different in every cellular clone (82). Hence, it was concluded that AAV integration, like that of all other animal viruses studied, occurred at many locations, if not completely at random.

We now know that this conclusion is incorrect, although the data are reproducible. The problem lies in the interpretation of the data. In particular, we did not appreciate that both cellular and viral sequences are rearranged during the integration process. In most latent infections in which virus can be rescued, the integrated DNA is present as a tandem array; presumably, at least one copy of the viral genome remains intact and in the original sequence arrangement.

Whether or not AAV integration is site-specific could be resolved only when integrated viral sequences and the flanking cellular sequences were cloned from latently infected cells (66). Three clones of integrated viral sequences were isolated from the originally described clone of latently infected human Detroit-6 cells that had been sequentially characterized for over 100 passages. Detailed study of the three cloned DNAs led to several interesting, and in some cases unanticipated, results.

(1) Two of the clones represented junctions between viral and cellular sequences. In one case the junction occurred approximately in the middle of the *itr* at the left end of the viral DNA. The other case was more complex,

representing several rearrangements of the viral sequence very close to the junction. The presence of sequence rearrangements at the junction immediately suggested that the original conclusion concerning site-specificity of integration needed to be reconsidered.

(2) A third DNA clone that represented a restriction fragment originally thought to contain a virus–cell junction turned out to be a tail–tail junction of AAV sequences with the *itr*'s deleted at the junction.

Fortunately, subclones of the flanking cellular sequences isolated from the two clones containing virus/cell junctions proved to represent single-copy sequences in the genome. When used to probe *Bam*HI genomic digests of uninfected cells, each of the flanking sequence subclones hybridized to a single fragment. However, when similar genomic digests from the original clone of latently infected cells were assayed in the same way, each of the probes hybridized not only to the single fragment observed for uninfected cells, but also to one or two additional fragments. The new observed fragments from latently infected cells were thought to represent disruption caused by integration of AAV DNA. The continued presence of fragments of the original size was thought to represent remaining copies of the original (i.e., wild-type) cellular sequence. This notion was supported by the observation that an AAV DNA probe hybridized to the new fragments, but not to the original. If this notion was correct, the way was cleared for a critical assessment of the issue of site-specific integration.

Genomic DNAs from 21 additional independently derived clones of latently infected cells (from three different laboratories) were digested with *Bam*HI, and Southern blots were probed with the flanking sequences from the original latently infected cell clone (*67*). If integration in the 21 new clones had occurred at the same site, more than one *Bam*HI fragment would hybridize to the probe; if integration had occurred at another site, the probe should have hybridized only to the fragment containing the uninterrupted original sequence. For 14 of the 21 clones, additional bands were noted, suggesting that in about two-thirds of the clones AAV had integrated in a site-specific manner. All the clones had retained a copy of the wild-type fragment. AAV-specific probes hybridized to a majority of the new fragments (22 of 36), but not to the wild type. As controls, equal numbers of genomic DNAs from uninfected lines of continuous cells and also from various lots of primary cells were tested in the same manner. There were no cases of additional fragments (i.e., no evidence for an RFLP involving the flanking sequences). From this set of experiments, we concluded that, in most cases of latent infection of continuous human cell lines, the AAV had integrated in a site-specific manner.

These results have been confirmed independently (*69*, *70*). By placement of the bacteriophage λ repressor binding site at a nonessential position in the AAV genome near the right end, the investigators could use purified λ

repressor protein to selectively isolate restriction fragments of genomic DNA from latently infected cells that contain integrated AAV sequences, and subsequently determine junction sites and flanking sequences. They have also found that in most, if not all, of the clones they examined, the AAV sequences had integrated into the specific integration site originally determined. The integration was not identical at the nucleotide level, but all the events appeared to be within a 100- 200-nt region. Half of the junctions occur within the *itr* and the other half are in the unique sequence upstream of the p5 promoter. This region has a high potential for hairpin formation (*83*).

Although the evidence for a highly preferred site of integration in continuous lines of human cells is strong, it should be noted that AAV can integrate into other sites in the human genome and into genomes that do not contain the specific site (e.g., the mouse genome). Some cases of alternative sites of integration in latently infected human cells have been reported (*84*) and we do not have conclusive evidence for site-specific integration in primary human cells. In particular, one experiment should be noted (*83*). Latently infected human cells were superinfected with AAV vectors containing a selectable marker. Doubly latently infected cell clones were isolated and characterized. Although the initial wild-type AAV had integrated into the specific site, the superinfecting genomes had integrated elsewhere. This may reflect a requirement for at least one intact copy of the integration sequence for continued cell viability (*83*).

B. Pre-integration Site

The location of the specific integration site was initially determined by hybridization to genomic DNAs isolated from mouse/human hybrid cell lines that contained only one or two human chromosomes. The probe was a 4.5-kb fragment cloned from uninfected cells that hybridized to both of the original flanking sequence probes. The results unambiguously assigned the integration site to human chromosome 19. This experiment was possible not only because the flanking sequences were single-copy, but also because the sequence was not detectable in the mouse genome under the conditions of hybridization stringency employed.

The localization of the integration site to chromosome 19 was confirmed and further refined by direct staining of metaphase spreads with biotinylated probes (*68*). The site was mapped to 19q13.3–qter in peripheral blood lymphocytes from normal volunteers. The same site was determined in HeLa cells, even though rearrangement of chromosome 19 in uninfected cells was noted. The integration site still localized with a marker specific for 19q. Latent infection did not further alter chromosomal morphology and integrated AAV sequences colocalized with the cellular sequences of the integration site.

A 4-kb fragment of cellular DNA containing *aavS1* (the AAV integration site) was cloned and sequenced (*71*). The overall (G+C)-content is 65%, but that of the 5′ 900 bases is 82% G+C. The sequence in this region meets the definition of a CpG island. The first 900 bases and the 3′ 1500 bases are rich in direct sequence repeats. Based on the criterion of an 11 of 12 match, there is one repeat for every 45 bases in the first 900 bases and one repeat for every 70 bases at the 3′ terminus. The middle section of the sequence has one repeat for every 220 bases, which is close to the expected probability. Near the 3′ terminus there is a tandem array of 10 copies of a minisatellite 35-mer. This minisatellite is known to occur at approximately 60 sites in the human genome, all of which are on 19q (*85*). There is a region of open reading frames in the middle of the sequence, and there is evidence for transcription of the region at a low level in several tissues and myeloid tumor cells, using reverse-transcription polymerase chain reaction. Thus, the sequence may have a relatively open conformation in some kinds of cells. The overall sequence has no significant homology with the AAV genome, although the *itr* and the 5′ 900 bases of the integration site (*aavS1*) do have in common a high (G+C)-content.

A significant question is whether *aavS1* serves as a specific integration site because of its sequence per se, or whether the specificity is determined in large part by the chromosomal location of the integration site. To test this, *aavS1* has been inserted into an Epstein–Barr virus (EBV)-based shuttle vector (courtesy of W. Sugden) which can replicate as a stable episome in human cells (using its own EBNA1 protein and the latent EBV *ori*) and can also replicate in *E. coli* (A. Beaton and K. I. Berns, unpublished data). Human 293 cells (transformed by adenovirus 5H) which also expressed EBNA1 were transfected with the shuttle vector carrying *aavS1*. Once cells carrying the vector were stably transformed, the cells were infected with AAV. After 1 or 2 days, episomal DNA was recovered by Hirt extraction (*85a*) and used to transform *E. coli*. Subsequently, bacterial colonies carrying the EBV vector were tested to see whether AAV sequences were present. Indeed, they were present in vectors carrying the *aavS1* sequence, but not in control vectors (C. Giraud, M. Linden, E. Winocour and K. I. Berns, unpublished data). Therefore, it seems clear that the *aavS1* primary sequence is a major determining factor in site-specific integration.

C. The Integrated Genome and Flanking Sequences

The sequences found flanking the junctions with AAV DNA have been compared to the cellular sequence *aavS1* prior to integration (*71*). There is evidence for inversion and rearrangement of the cellular sequence as a consequence of recombination. One virus–cell junction occurs at nts 1144–1147 of the cell sequence, which continues to nt 1600. The other virus–cell

junction is at 1027–1030, and the cell sequence continues up to 1141–1144, where there is a cell–cell junction going abruptly to nt 3700; the sequence then continues back down. Thus, the sequence 1027–1141 has apparently been inverted (and translocated) and the same nts 1141–1147 are involved in two recombinational events. As described above, at one of the junctions, the viral sequences have been rearranged as well. From the *Bam*HI site at nt 1045, the sequence is uninterrupted down to nt 394 (where there is an unidentified 5-bp insertion). The AAV sequence resumes at nt 1278, but on the opposite strand. The sequence continues up to nt 4591 in the *itr*, where there is another strand switch. It is impossible to identify unambiguously the bases at the crossover point, as nts 273–267 are identical to nts 4585–4591; the sequence continues from nt 273 to nt 153. This makes it seem that there was a concerted complex breakage and reunion type of recombination. At all three junctions there was a 4- or 5-base homology, but no more. Two of the junctions have possible topoisomerase-I cleavage sites nearby and two of the junctions can form a hairpin structure similar to one of the cross-arms in the AAV terminal T-shaped hairpin. In summary, *aavS1* has numerous features that might predispose to recombination per se, but it is difficult to attribute the observed site-specificity to the features described.

The specificity seems likely to be at least partly attributable to the virus as well. One feature that may be important is the folded conformation of the *itr*. Apt to be at least as important are the larger Rep78 and Rep68 proteins. These can bind specifically to both the *itr* (*50*) and *aavS1* (the integration site) (*85b*). Rep78 and Rep68 can function as DNA helicases (*51*) and a site-specific DNA "nickase" (*52*), activities likely to be important in integration. Construction of AAV vectors missing the *rep* gene leads to decreased specificity of the site of integration (*86*). However, the efficiency of transduction increases remarkably, which suggests an important role for the *itr*, as it is the only remaining AAV genome component. In other experiments, the presence of the *rep* gene in *trans* seemed to heighten the specificity of integration (*67*, *82*).

V. Consequences of Latency

Studies of cells infected in the absence of helper virus have revealed a number of changes in the cellular phenotype. A variety of cell types infected with AAV display decreased plating efficiency and evidence of cell-cycle arrest (*18*, *87*, *88*). Cells latently infected with AAV have increased sensitivity to a variety of genotoxic agents (e.g., UV, MNNG) (*75*, *84*, *89*, *90*). Such treatments are known to induce a state of permissiveness for AAV DNA replication (*91*).

Experiments with the human leukemic cell line HL60 and immortalized

keratinocyte lines (HaCaT and HPK Ia) infected with AAV resulted in reduced cell growth and detectable levels of differentiation-associated antigens, as well as altered expression of several oncogenes (c-*myc*, c-*myb*, and c-*fos*) (*92*). Other studies noted changes in the matrix attachment properties of normal cells (*93*).

It is unclear exactly how these various effects are mediated. One study showed that UV-inactivated AAV is indistinguishable from wild-type AAV in the ability to induce cell-cycle arrest (*87*), while another showed the *rep* gene to be required for enhanced UV sensitivity and inhibition of gene amplification (*90*).

VI. Anti-oncogenesis

In addition to the mechanisms by which AAV enters into and is activated from the latent state, the effects of AAV latency on the phenotype of the host cell have been the subject of much study. All nuclear DNA viruses establish persistent infections, but AAV is the only one known that does not cause oncogenic transformation. Rather, AAV has been characterized as anti-oncogenic. AAV infection prevents cellular transformation after transfection with oncogenes and after infection with DNA tumor viruses (*12–20, 94*). At this level, the *rep* gene has been implicated as the causal agent of inhibition. AAV also appears to reverse the transformed phenotype of cells (*84*).

In our own experiments with adenovirus-5-transformed hamster cells, anchorage independence and saturation density, but not viability, were affected by AAV infection (*16*). Interestingly, the oncogenicity of cells injected into newborn hamsters was reduced to 10–25%. In this case, at the molecular level, the effect seemed to be at a posttranscriptional level; the 55-kDa E1b protein was reduced by the same percentage observed for the decrease in oncogenicity.

Other experiments have involved infection of newborn animals with adenovirus (*12, 14*). Coinfection of AAV with adenovirus blocks tumor formation in susceptible animals. In a newborn animal experiment with defective interfering particles, an effect was also seen (*17*). For this reason, the *itr*'s were hypothesized to be responsible for the anti-oncogenic effect. Further support for this possible mechanism has not been reported.

VII. The Permissive State

In culture, a cell can be rendered permissive by several treatments. The most common is infection by adenovirus, but infection by herpesviruses has the same result. Although not as effective, several genotoxic or stressful

environments also render at least some cells positive for virus production (*95, 96*) or DNA replication (*75, 97–101*) or transcription. Simply heating HeLa cells to 40°C dramatically increases the levels of AAV transcripts seen in the absence of helper coinfection (*74*). Treatment of cells with a variety of metabolic inhibitors or DNA-damaging agents turns on DNA replication and, in some cases, virus production.

Genetic studies have been done to determine the helper functions supplied by adenovirus and herpes simplex virus. The adenovirus E1 genes, E2a, E4, and VA RNAs are all involved in supplying helper function (*102, 103*). Each of these affect gene expression in a general way in the cell. E2a is also required for adenovirus DNA replication, but does not appear to participate in AAV DNA replication.

Similarly, early herpes simplex genes are required for helper function. However, several genes directly involved in DNA replication also seem to be involved, albeit there is some disagreement between the findings of two laboratories as to the specific DNA replication genes implicated (*104, 105*). The requirement for DNA replication genes in this instance may reflect an earlier finding that AAV replication complexes contain cellular DNA polymerase when adenovirus is the helper, but contain the herpes DNA polymerase in the case of a herpesvirus helper (*106*). With this exception, AAV DNA replication seems to involve primarily cellular proteins and Rep78 and/or Rep68.

The primary helper virus functions required are those that affect gene expression; the various genotoxic treatments also have the effect of turning on repair genes. In some notable instances the same genes are turned on by different stimuli; to wit, adenovirus E1a turns on some cellular heat-shock proteins (*107*).

As a consequence of the various ways in which a cell may be made permissive for AAV gene expression and DNA replication, we have come to the conclusion that AAV is a virus that is programmed to establish a latent infection in a healthy cell. Only when the cell has been stressed by the environment and repair genes have been turned on does the cell become permissive for AAV rescue and replication. In this way, when the host cell is no longer in a good position to assure the perpetuation of the AAV genome, the viral genome is activated and virus is produced to infect new cells. This is a life cycle similar to that first discovered for the bacteriophage λ (*108*). For this reason we believe that it is incorrect to consider AAV a defective virus; instead, it should be considered a virus adapted to a latent life style.

The role of the *rep* proteins in the establishment of the latent state has already been described. They are of equally central importance in the productive phase of the viral life cycle. Under permissive conditions, all three of the AAV promoters are *trans*-activated by the larger *rep* proteins (*109*). What

is striking is the inhibition by the *rep* proteins of the expression of the early genes of helper adenovirus. All adenovirus early gene expression is inhibited by *rep*, including E1a, which *trans*-activates expression of the *rep* gene. For this reason, care must be taken in an AAV + adenovirus coinfection with respect to the relative multiplicity of infection of the two viruses. If too much AAV is present, *rep* is so effective in shutting off adenovirus expression that the cell does not become permissive for AAV-productive infection. Under optimal conditions of MOI ratio to maximize AAV production, the yield of adenovirus is severely reduced (down to 10%) (*4, 110–112*). This is a further indication of the predilection of AAV for establishing a latent infection and is reminiscent of bacteriophage λ, in that high MOI predisposes to the establishment of lysogeny.

In addition to its effects on gene expression, *rep* also plays a major role in AAV DNA replication. As stated, the two larger *rep* proteins are required for initiation of AAV DNA synthesis and the production of duplex replicative intermediates. The smaller *rep* proteins appear to function in the removal of mature virion single strands from the replicative intermediates and the encapsidation of the DNA (*113*), possibly in a concerted reaction. *Rep* appears to be toxic in some cells (*75*), but it is difficult to assess this in a productive infection because of the normal toxicity of adenovirus.

VIII. Rescue and Replication

To perpetuate its genome, AAV must rescue itself from the integrated state and cause it to undergo a productive infection. The rescue process has been studied using a model system that appears likely to reflect a physiological process. As mentioned earlier, a recombinant plasmid containing the linear duplex form of AAV DNA inserted into a bacterial vector, such as pBR322, is infectious. Transfection of this construct into permissive cells (infected by adenovirus) leads to rescue and productive infection. The system has been studied in detail and has yielded much information concerning the genetics and replication of AAV. At the genetic level it is clear that the *rep* gene must be intact for rescue and replication. The *itr* is also a key element in *cis*. Several observations have been made with respect to its role in the process. (1) A deletion within one *itr* can be rescued and repaired, presumably by use of the other *itr* as a replication repair template or as a substrate for recombination (*114*). If the deletion extends into the unique sequence, it is lethal. (2) Deletion of the terminal 55 bases (approximately half of the palindromic sequence) from both ends of the insert blocks rescue, but allows replication of the entire construct. Normally, only the rescued AAV, but not the pBR322 vector, is replicated. (3) Deletion of the terminal

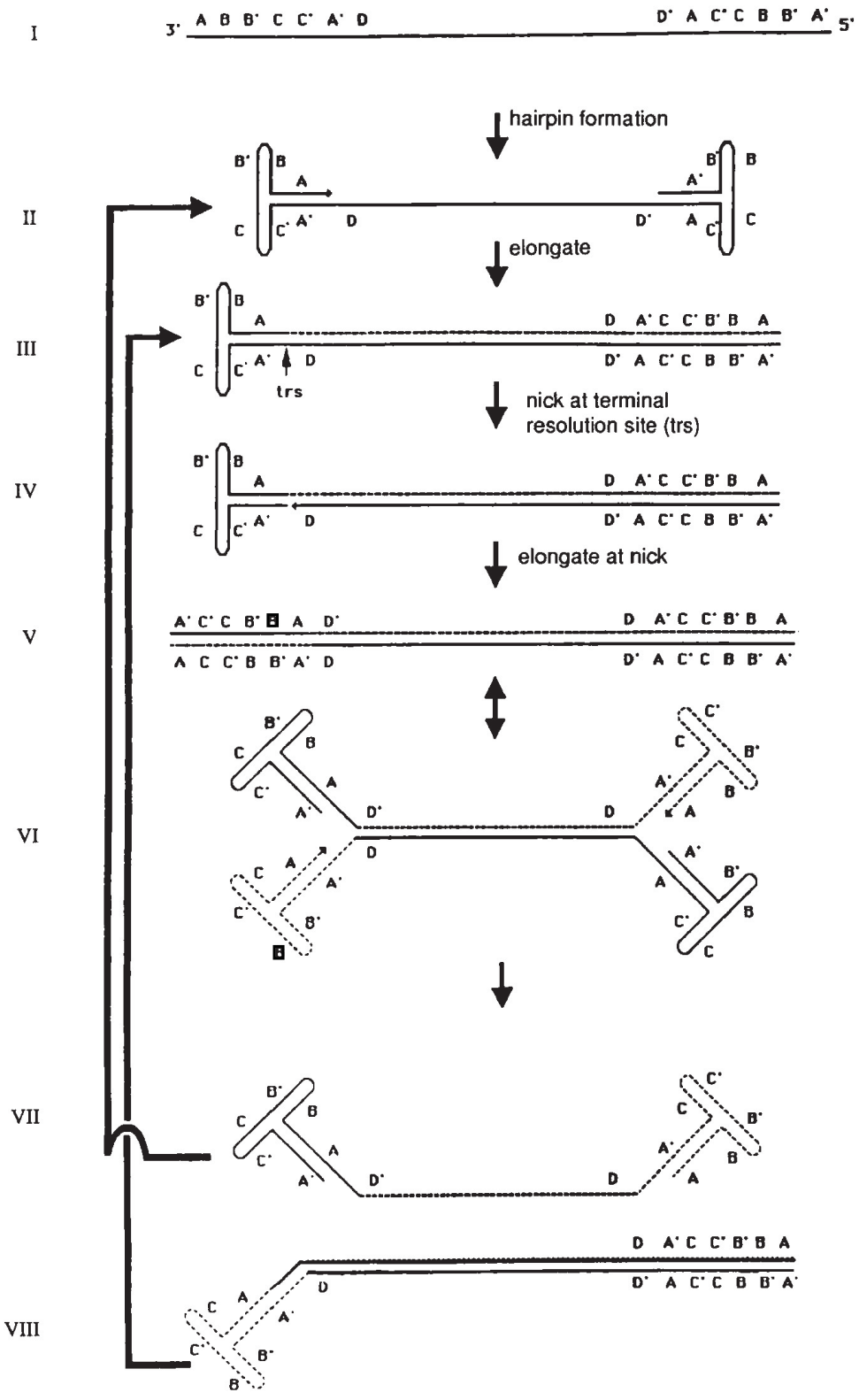
122 bases from both ends inhibits replication as well as rescue (A. Beaton, G. Biamonti and K. I. Berns, unpublished data).

The current model of AAV DNA replication is shown in Fig. 3 (*115–117*). The fundamental aspects include use of the terminal hairpin as the primer for replication, and elongation by a single-strand-displacement mechanism. No evidence for simultaneous synthesis of a complementary strand via Okazaki fragments has been found (*118*). The model predicts inversion of the symmetrical part of the *itr* to resolve the hairpin intermediate and maintain the full-length 5′ terminus of the genome (see Fig. 3). Because the *itr* is not perfectly symmetrical from nts 42 to 84, inversion should result in sequence heterogeneity in this region. Indeed, both sequences result from rescue of a clone with one initial *itr* sequence orientation (see Figs. 2 and 3). The model is in accord with all of the data from the plasmid transfection experiments described above (*40*).

However, to determine the proteins involved in the process, it is necessary to be able to work with an *in vitro* assay of DNA replication. This has now been achieved. Initial experiments were done with the AAV/SV40 chimera described earlier in which the p5 promoter was deleted and substituted by the SV40 regulatory region (*119*). Putting the construct into an *in vitro* SV40 DNA replication assay led to rescue of the AAV insert. The rescue products were linear duplex AAV DNA and linear duplex pBR322 DNA. Both linear duplex products were cross-linked at both ends by the symmetrical sequence of the AAV *itr*.

A model of the rescue process is shown in Fig. 4. The model assumes that the *itr*'s at the junctions between AAV and pBR322 sequences can form a cruciform structure, which is a conformation equivalent to a Holliday structure, the posited intermediate in homologous recombination (*120*). An activity is presumed to cut the structure across the base and to seal the break created, leading to covalent linkage of previously unlinked sequences. This is viewed as a reaction involving conformation rather than sequence specificity. Within limits, partial extrusion of the palindromic sequence to form the cruciform would lead to the same products, regardless of the specific site of cleavage and resealing. No viral gene products are required, since none are in the reaction, which includes extract from uninfected HeLa cells and SV40 T-antigen. Omission of T-antigen still leads to a reduced level of rescue, so replication is not essential. However, replication driven by either T-antigen

FIG. 3. The linear single-stranded genome of AAV is not naturally found in the conformation drawn in structure I. The ends of parvoviral genomes are resistant to the single-strand-specific endonuclease S_1, suggesting that they are usually in the partially duplex (hairpinned) conformation shown in structure II (*37, 139, 140*). In this conformation, the left hairpin can provide a 3′ hydroxyl group (indicated by an arrowhead at the end of the strand) for self-primed DNA replication.


I
3' A B B' C C' A' D
D' A C' C B B' A' 5'
hairpin formation
II
elongate
III
trs
nick at terminal
resolution site (trs)
IV
elongate at nick
V
VI
VII
VIII

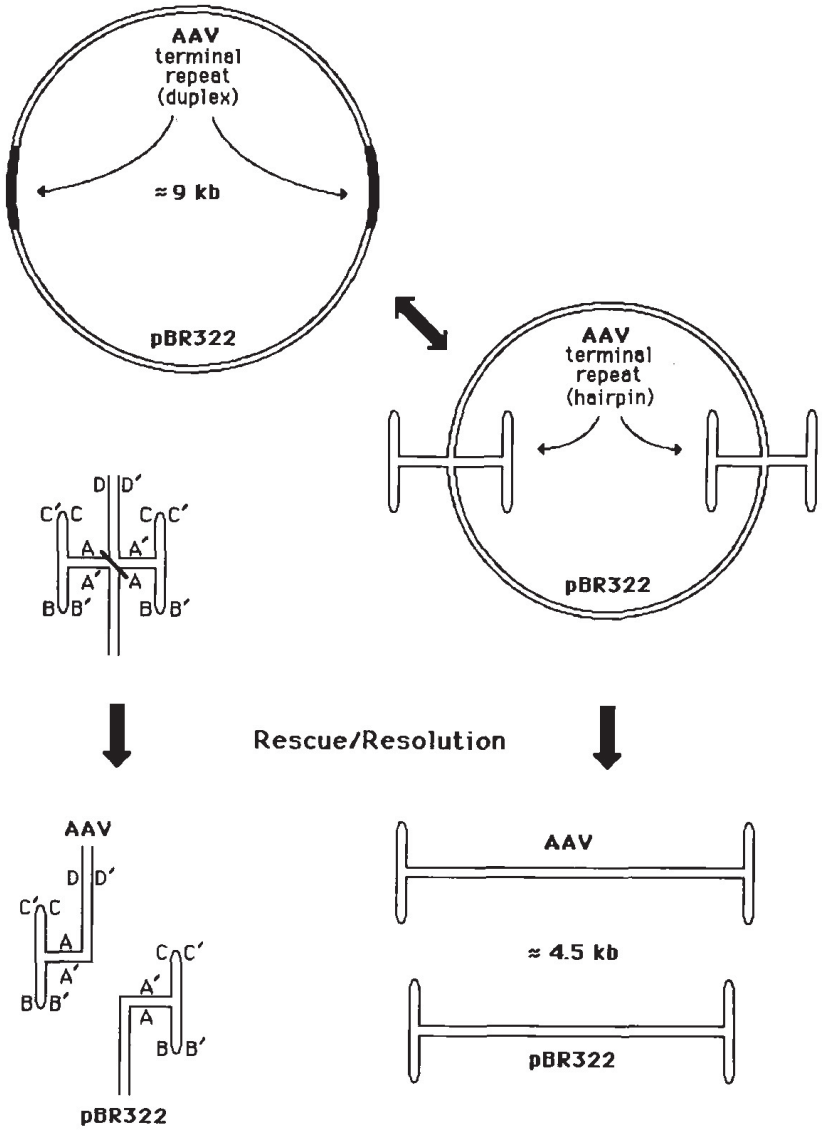


FIG. 4. The plasmid pSM620 is an infectious molecular clone of AAV in a pBR322 background. When used as a template in an *in vitro* DNA replication assay, factors present in an uninfected HeLa cell extract catalyze the cleavage of the 9.0-kb circular plasmid into the two ≈4.5-kb molecules diagrammed at bottom. The proposed mechanism for the rescue event shows similarities to the resolution of Holliday structures. The extrusion of the cruciforms would be favored by negative supercoiling.

or AAV Rep68 or Rep78 protein greatly enhances rescue, presumably by enhancing cruciform formation.

Subsequently, it was possible to develop an *in vitro* assay that combined rescue and AAV DNA replication. Previous attempts to set up an *in vitro* assay had been stymied because the terminal hairpin that the model assumes is used as a primer to initiate AAV DNA replication could be extended by any DNA polymerase. Hence, the physiological significance of DNA synthesis *in vitro* was uncertain.

With the observation of rescue of the hybrid AAV/SV40 insert from the vector in the SV40 assay, an *in vitro* assay for AAV-driven replication was established on the premise that any replication, possibly just initiation, would lead to a significant enhancement of rescue. In fact, it was possible to observe significant amounts of AAV DNA replication in an assay that faithfully displayed all of the properties seen *in vivo*. Initially, replication (as measured by resistance to *Dpn*I) occurred only when extracts from HeLa cells productively infected with AAV and adenovirus were used (*121*). Comparable extracts from uninfected HeLa cells or from cells infected with either AAV or adenovirus alone did not support replication. All extracts did cause rescue to occur, but as expected, the extract from the productively coinfected cell gave an increased level of rescue. Only the rescued AAV DNA was replicated; pBR322 sequences were not.

In vitro experiments with mutant constructs with deletions in the *itr* also gave results comparable to those seen *in vivo*. Deletion of the terminal 55 bases from both *itr*'s of the insert inhibited rescue, but did not inhibit DNA replication. In this case, both AAV and pBR322 sequences were replicated. Deletion of the terminal 103 or 122 bases from both *itr*'s blocked both replication and rescue. The results with the 55-base deletion mutant are of particular interest. A major question is whether rescue occurs first and the rescue product is then replicated, or whether replication initiates while the DNA is still in the integrated state. The results above certainly indicate that the latter is quite likely.

The *in vitro* assay has allowed a more detailed analysis of both the intermediates of replication and the proteins that may be required. The model for replication (Fig. 3) predicts that the terminal hairpins covalently linking the two strands after rescue will be resolved and extended. This has been observed among the replication products of the *in vitro* reaction. The model also predicts that in some cases, when the newly synthesized progeny strand reaches the 3′ end, the new *itr* will simply fold back on itself to serve as a primer for another round of synthesis, leading to a dimeric duplex replicative intermediate. These have been seen *in vivo* and have also been shown to occur in the *in vitro* reaction.

The differences between a productively infected cell extract and extracts

from other cells pose the question of which proteins are needed for the rescue and replication processes. In the first instance, the issue is which AAV products might be required, and then whether any adenovirus gene products are specifically needed *in vitro*. Finally, of course, is the major issue of which cellular proteins might be involved. Experiments in our laboratory and that of N. Muzyczka indicate that no viral proteins are required for rescue, although it is not certain that replication-enhanced rescue may not differ to some extent mechanistically. There is some difference with respect to the question of whether adenovirus provides any specific gene products required *in vitro*. We have used Rep78 expressed in a vaccinia expression system (C. J. Leonard and K. Berns, unpublished) and both Rep68 and Rep78 cloned in *E. coli* (P. Ward, R. Kotin, B. Safer and K. Berns, unpublished), and have been able to observe replication *in vitro* using extracts from uninfected HeLa cells. Muzyczka's laboratory has used Rep78 and Rep68 expressed in a baculovirus system and has not been able to observe replication using an uninfected HeLa cell extract, but has had good success with extracts from cells infected by adenovirus (N. Muzyczka, personal communication). The reasons for the difference are not clear.

IX. Adeno-associated Virus as a Potential Gene-Therapy Vector

Shortly after infectious clones of AAV became available, the possibility of using AAV as a vector for introducing foreign genes into cells was explored. Studies using a neomycin-resistance marker gene in place of the capsid gene showed promising levels of transduction in Detroit-6, HeLa, and 293 cells (*122–125*).

Later experiments using DNA fragments cloned between the AAV *itr*'s showed that the *itr*'s alone were sufficient *cis*-acting signals for packaging and integration (*126, 127*). This allows for insertion of genes up to 5 kb in length in recombinant AAV viral vectors. The stable integration of AAV DNA in latent infection may be a desirable attribute in a gene-therapy vector. Unlike retroviruses or adenoviruses, AAV has never been associated with oncogenic events due to the insertion of viral regulatory elements. Additionally, the possibility of targeting vector insertion to the *aavS1* sequence could more clearly define the risks of AAV-based gene therapy.

AAV vectors have been tested in a wide variety of cell types (*128–130*) introducing an assortment of genes. AAV vectors have expressed anti-HIV antisense RNA in A3.01 cells (*131*) and HIV envelope genes in 293 cells (*132*). They have also been successfully used for β-globin expression in 293 cells (*133, 134*) and γ-globin expression in K562 erythroleukemia cells (*135*).

One interesting set of experiments has addressed the feasibility of using AAV vectors for introducing genes into cultured airway epithelial cells (IB3-1) from a patient with cystic fibrosis (*136*). Further work succeeded in correcting the CFTR defect of these cells in culture (*137*, *138*). In light of reported complications in clinical trials of adenovirus-based gene therapy for cystic fibrosis, AAV-based vectors may prove to be an important alternative.

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