FEBS Letters 407 (1997) 78–84 FEBS 18456

The packaging capacity of adeno-associated virus (AAV) and the potential for *wild-type-plus* AAV gene therapy vectors

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Received 20 February 1997

Abstract Because of its ability to integrate chromosomally and its non-pathogenic nature, adeno-associated virus (AAV) has significant potential as a human gene therapy vector. Here we investigate the maximum amount of DNA which can be inserted into the AAV genome and still allow efficient packaging into an infectious virus particle. Altered wild-type AAV genomes were constructed with inserts, which increased in size by 100 bp, ligated at map unit 96. These large wild-type-plus genomes were able to replicate and produce infectious virus, at levels slightly reduced but comparable to normal sized wild type, until the insert size reached 1 kb. These data indicate that the maximum effective packaging capacity of AAV is approximately 900 bp larger than wild type, or 119%. Furthermore, it is demonstrated that these large AAV genomes are able to latently infect cells by chromosomal integration as does wild-type AAV. These data suggest that therapy vectors carrying a foreign gene of 900 bp or less can be generated from AAV, by ligation into non-essential locations, and result in a recombinant AAV virus with a fully wild-type phenotype. Such wild-type-plus AAV vectors will have both advantages and disadvantages over defective recombinant AAV virus — the most important advantages being the ease in which high titers of infectious virus can be generated and the ability to specifically integrate within chromosome 19. Once the concern subsides over the presence of wild-type AAV in clinical applications, wild-type AAV vectors may find specific application niches for use in human gene therapy.

Key words: Adeno-associated virus; Gene therapy; Packaging capacity

1. Introduction

Adeno-associated virus (AAV) is a helper-dependent human parvovirus which is able latently to infect cells by chromosomal integration. Various studies, from 1970 to 1986, demonstrated that 15–30% of immortalized cells could be latently infected with wild-type AAV and that the AAV genome was chromosomally linked [1–3]. After the mapping of AAV genes and their functions [4,5], a similar ability was demonstrated for recombinant AAV in 1984 in immortalized tissue culture cells [6,7], and in 1988 recombinant AAV transduction of primary hematopoietic stem cells was achieved [8]. More recently, in 1990, the preferred site of wild-type AAV integration was demonstrated to be in a region of human chromosome 19 [9–11]. Many laboratories have confirmed and extended these data and demonstrated the utility of AAV-based vectors [12–18]. In the last year the likely initiation of the deciphering of the mechanism of wild-type AAV integration has begun with the finding that a complex between chromosome 19 DNA and AAV terminal repeat DNA was formed in the presence of the AAV Rep78/68 protein [19–21], the same protein which is required for AAV DNA replication [4,5].

AAV's largest disadvantage as a gene therapy vector may be its limited packaging capacity, dictated by the limited size of the AAV virion. Additional disadvantages of recombinant AAV as gene therapy vectors include the difficulty in the producing of high virus titers and their inability, because they lack the *rep* gene (Rep78/68), to target integration into chromosome 19. In this study all of these problems are addressed. To address AAV's packaging capacity large *wild-type* AAV genomes, which increase in size in steps of 100 bp, were utilized to investigate AAV's maximum capacity. It is demonstrated that genomes larger than wild type by 900 bp, or 119%, have a fully wild-type phenotype, including chromosome 19 integration. Thus, genes of significant size might be inserted into AAV and still result in a phenotypically wild-type virus. These findings also address the *titer* and *specific integration* problems as the use of *wild-type* AAV vectors would circumvent both of these limitations of recombinant, defective AAV vectors.

2. Material and methods

2.1. Cells and plasmids

HeLa, SW13, and D510 (Detroit 510) cells were grown in Delbecco's Modified Eagles Medium with 7% fetal bovine serum plus penicillin and streptomycin. The construction scheme of ins96-λ-R, ins96-λ-M, and ins96-λ-F has been described previously [6]. Briefly, to generate these plasmids random *Sau*3AI fragments from λ phage were ligated into the *Bgl*II site of ins96. Ins96-λ-R, ins96-λ-M, and ins 96-λ-F have insets of 0.5, 1.1 and 2.8 kb, respectively. To generate the 100 bp stepped large AAV plasmids '100 bp DNA ladder' marker DNA was purchased from Gibco BRL (catalog #15628), agarose gel electrophoresed, and the 600, 700, 800, 900, and 1000 bp fragments were cut out of the gel, and isolated by the Quiex DNA isolation kit (Qiagen). Each of the isolated fragments was ligated into the *Bgl*II site of ins96 [4] using *Bam*HI linkers to generate ins96+0.6, ins96+0.7, ins96+0.8, ins96+0.9, and ins96+1.0.

2.2. DNA replication and virus production

The AAV genomes were assayed for their ability to generate infectious virus using a sequential, two plate, Southern blot analysis [4–6]. After AAV plasmid transfection plus Ad infection, half of the cells from the first plate are assayed for DNA replication by Southern blot. The medium and the other half of cells from the first plate (now a potential virus stock) are then added to a second plate to assay for virus infection and DNA replication, again by Southern blot analysis. AAV constructs which are unable to produce infectious virus, such as capsid (*cap*) mutant ins63, are unable to survive and replicate in the second plate [[4–6], and Fig. 2]. In more detail, 1 μg of AAV plasmid was DEAE/dextran transfected into the indicate cells (10 cm plate at 80% confluence) as described previously [6]. The cells were then infected with adenovirus type 2 at an multiplicity of infection (MOI) of 5. At 36–48 h later one-half of the cells was lifted off the plate by a

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PII S0014-5793(97)00311-6

rubber policeman and the cells and medium were removed. The remaining cells (first plate) were analyzed by low molecular weight Hirt DNA extraction [26], and Southern blot analysis (10% of DNA isolated)[27], probing with ^{32}P-labeled AAV DNA or ^{32}P-100 bp ladder DNA as indicated to observe viral replication. The half cells scrapped off and left in suspension in DMEM (10 ml) were freeze/thawed 3 times, heated to 56°C for 30 min (to kill Ad), and one-third (33%) of the suspension (potential virus stock) was then added to a second plate of tissue culture cells which were subsequently infected with Ad2 at an MOI of 5. At 36–48 h the second plate of cells were Hirt DNA extracted (virus production). 10% of the extracted DNA from both the first and second plates were agarose gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled AAV DNA or ^{32}P-100 bp ladder DNA probe. 'dsm' indicates double-stranded monomer DNA, and 'ssm' indicates single-stranded monomer length DNA.

2.3. Construction of latently infected cells

10^3 HeLa or D510 cells was infected with 10^6 infectious units of ins96+0.9 virus. The cell cultures were then grown for 4–5 weeks, with at least three cell splits/replatings.

2.4. PCR amplification and dot-blot hybridization analysis

Two different PCR procedures, amplifying either the AAV *rep* gene or the AAV TR–chromosome 19 S1 junction, were performed on DNA from the indicated D510 cells. PCR amplification and dot-blot hybridization analysis for AAV *rep* sequences has been described [28]. The primers for PCR amplification of an AAV TR–human chromosome 19 junction have been described [10]. Each PCR reaction contained between 1 μg of total DNA isolated from the indicated cell. The reaction buffer, with and without pSM620 (cloned wild-type AAV, 1 pg pSM620 in 1 μg of HeLa cell genomic DNA), served as the positive and negative controls, respectively, for the AAV *rep* amplifications. One-tenth volume of the PCR product was then denatured, fixed to a nylon membrane and hybridized with the appropriate internal ^{32}P-probe (sequences located between the two primers). The AAV–human chromosome 19 junction product was probed and identified using two different probes. One probe, used in Fig. 7C, was of sequences of the AAV terminal repeat, from nt 95–125. The other probe, used in Fig. 7D, was of human chromosome 19 sequences upstream of, but not overlapping with, the chromosome 19 side PCR primer (5′-ACTCCAATGCGGAAGAGAGTAGGTCG).

3. Results

3.1. Large AAV genomes with insertions of 1.1 kb and larger are severely defective for producing infectious virus

To investigate the packaging capacity of AAV, various large AAV genomes were assayed for their ability to generate infectious virus. The location of the DNA insertions is shown in Fig. 1. For one series of large AAV genomes, *Sau*3AI fragments from bacteriophage λ were ligated into the *Bgl*II site of ins96 to generate a series of large AAV genomes [6]. As no *cis* or *trans* elements are located at map unit 96, thus these large AAV genomes are genetically wild-type and are simply larger than normal size. Three resulting clones, ins96-λ-R (+0.5 kb), ins96-λ-M (+1.1 kb), and ins96-λ-F (+2.8 kb), were used for analyzing the packaging capacity. These large AAV genomes

Fig. 1. Structure and *Pst*I map of the large AAV genomes. The phenotypic map of AAV is shown. At the top are indicated the regions attributed to the four major phenotypes (TR/*ori, rep, lip, cap*) labeled [4]. Also indicated is the position, a *Bgl*II site at map unit 96, into which foreign DNA was ligated to generate the larger than wild-type AAV vectors. Heterologous DNA was inserted here because no *cis* or *trans* elements are located here. Below is shown the *Pst*I restriction sites within the cloned AAV plasmid ins96 from which the larger than wild-type AAV vectors were generated, and the resulting fragment sizes.

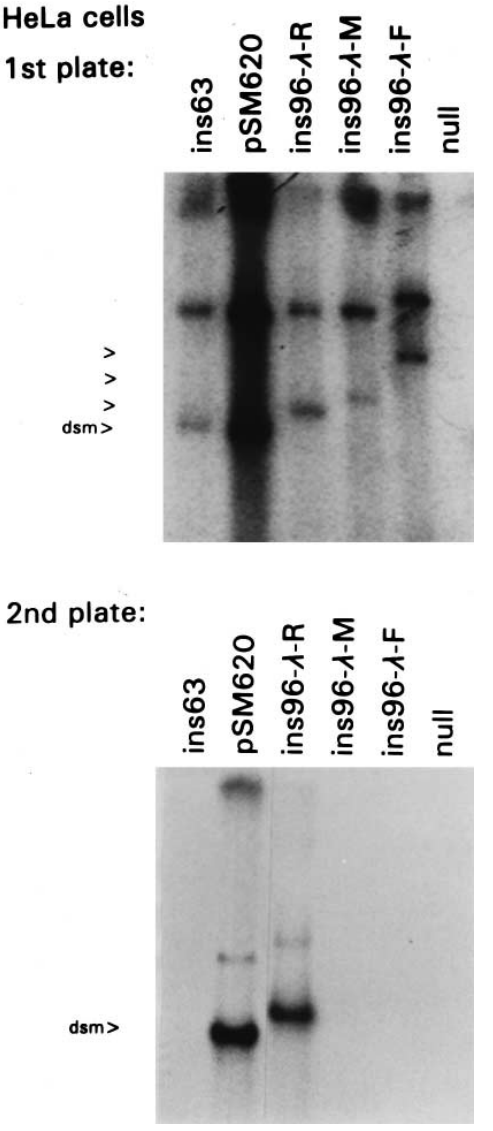

Fig. 2. Genetically wild-type AAV genomes with inserts of 1.1 kb and larger are severely defective for producing infection virus. Various AAV genomes were assayed for their ability to generate infectious virus. Shown is a sequential, two plate Southern blot analysis as described in detail in Section 2 (The first plate assays for DNA replication by Southern blot.) Materials from the first plate, a potential virus stock, are then added to the second plate. Thus, Southern blot analysis of the second plate assays for virus infection and DNA replication. The probe was ^{32}P-labeled AAV DNA. Note that wild-type pSM620 is able to generate infectious virus which is able to infect and replicate in the second plate. In contrast, ins63, a *cap* mutant, is unable to produce infectious virus in the first plate and is unable survive to replicate in the second plate. Also note the ins96-λ-R (+0.5 kb) is able produce infectious virus, while ins96-λ-M (+1.1 kb) and ins96-λ-F (+2.8 kb) are defective in virus production. As these genomes are genetically wild type the inability to produce infectious virus is likely due to the increase in genome size. No *cap* mutant has ever been found to survive and replicate in the second plate.

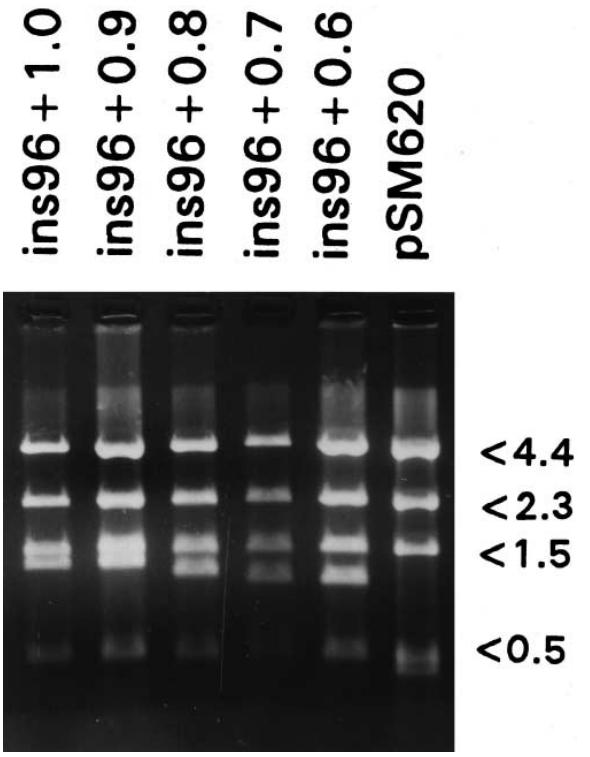

Fig. 3. *Pst*I restriction analysis of the large AAV genomic plasmids. Shown is a *Pst*I restriction analysis of the plasmid DNA of larger than wild-type AAV cloned genomes. One ug of each of the indicated AAV plasmids was analyzed by *Pst*I digestion, agarose gel electrophoresis, and ethidium bromide staining. The *Pst*I sites of the AAV genomes and plasmids are shown in Fig. 1. Note that the *Pst*I DNA fragment containing the right AAV TR increases in size with the increasing size of the DNA insertion. Originally the right TR *Pst*I fragment was 0.45 kb in size as shown in the pSM620 digestion (fully wild-type AAV). These altered *Pst*I fragments increase in size by 0.6–1.0 kb depending upon the size of the DNA ligated into the *Bgl*II site at map unit 96.

were then compared to wild-type AAV (pSM620 [22] and ins63 [4]. Ins63 is an 8 bp *Bgl*II linker insertion frame shift mutant at map unit 63, within the capsid gene (*cap*), and is able to replicate (*rep*+), but unable to produce infectious virus (*cap*−).

A sequential, two plate Southern blot analysis [4–6] was used to observe virus production, shown in Fig. 2 (described in detail in Section 2). To carry out this analysis the AAV genomes, as plasmids, were DEAE/dextran transfected into an initial, first plate of Ad-infected HeLa cells. At 2 days post-transfection low molecular weight Hirt DNA was isolated from half the cells, then 10% of the DNA was gel electrophoresed, Southern blotted, and probed for AAV sequences (top of figure). The second half of the cells plus the medium, now a potential AAV virus stock, was used to infect a second plate of Ad-infected cells. At 2 days post-infection low molecular weight Hirt DNA was isolated from second plate, then 10% of the DNA was gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled AAV sequences (bottom of Fig. 2).

As shown in Fig. 2, as expected, wild-type AAV, pSM620, was able to generate virus in the first plate and infect and replicate in the second plate (bottom of Fig. 2). In contrast, the *cap* mutant ins63 was able to replicate its DNA in the first plate, but was unable to produce infectious virus and to survive to replicate in the second plate. No AAV *cap* mutant has been found to survive and replicate in the second plate of this assay [4,6]. Of the large AAV genomes, Ins96-λ-R (+0.5 kb) was found to be able to produce infectious virus, and at a level comparable with wild-type pSM620. In contrast, both ins96-λ-M (+1.1 kb) and ins96-λ-F (+2.8 kb) were severely defective in virus production. As these genomes are genetically wild type, the inability to produce infectious virus are likely due to the increase in genome size. However, on long exposure a small amount of ins96-λ-M was found to be replicating in the second plate (about 0.1% of wild type) indicating that it was packaged at a very low level. In several experiments ins96-λ-F was never found to replicate in the second plate. In this particular experiment pSM620 replicated slightly better in the first plate than the large ins96-based AAV genomes. This difference is due to experimental variability and is not seen in other similar experiments (e.g. Fig. 5) These data indicate that the maximum efficient packaging capacity for the

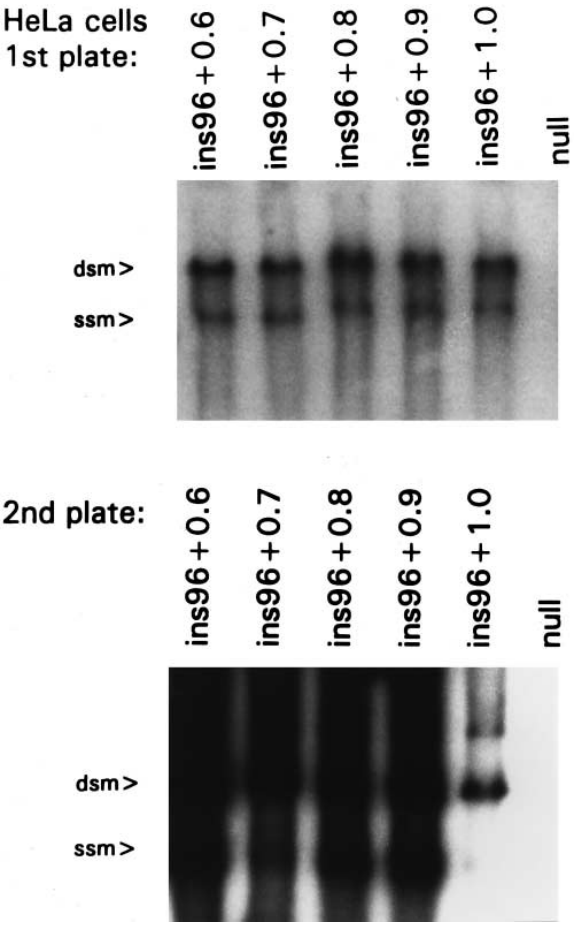

Fig. 4. Ins96+1.0 is packaged at a lower efficiency than smaller genomes in HeLa cells. Shown is a sequential, two plate Southern blot analysis for infectious virus production, similar to that described in Fig. 2. In this experiment, however, the stepped 100 bp large AAV genomes are analyzed and ^{32}P-labeled 100 bp ladder [insert] probe) is used to score for replicating AAV DNA. Note that all the AAV plasmids were able to replicate in the first plate; however, in the second plate ins96+1.0 was defective infectious virus production and subsequent DNA replication, when compared to the slightly smaller AAV genomes. These data indicate that the maximum packaging capacity of the AAV virion was exceeded by ins96+1.0. 'dsm' indicates double-stranded monomer DNA, and 'ssm' indicates single-stranded monomer length DNA.

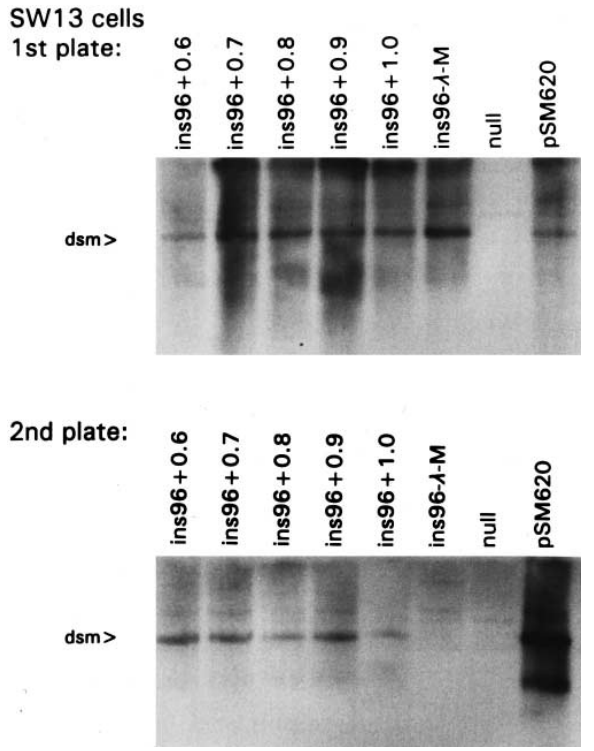

Fig. 5. Ins96+1.0 is packaged at a lower efficiency than smaller genomes in SW13 cells. Shown is a sequential, two plate Southern blot analysis for infectious virus production, similar to that described in Figs. 2 and 4. In this experiment, however, the stepped 100 bp large AAV genomes are analyzed in SW13 cells and ^{32}P-labeled AAV DNA is used to score for replicating AAV DNA. Note that all the AAV plasmids were able to replicate in the first plate; however ins96+1.0 was significantly reduced for significant replication (could not effectively produce infectious virus) in the second plate. Ins96+0.9 was at 70.5±32%, and ins96+1.0 at 25.7±16.2%, the level of pSM620 in the second plate as determined by densitometric analysis in three experiments. There are a few faint bands in the ins96-λ-M and null lanes of the second plate analysis. These are high non-specific bands, probably due to contaminating high molecular weight genomic DNA and incomplete washing of the membrane. These bands were not observed in any of the redundant experiments. With Fig. 4, these data indicate that the maximum packaging capacity of the AAV virion was exceeded by ins96+1.0. 'dsm' indicates double-stranded monomer DNA, and 'ssm' indicates single-stranded monomer length DNA.

AAV virion was somewhere between 0.5 and 1.1 kb larger than wild type (4.7 kb).

3.2. *A 900 base insertion is the maximum size allowable for near wild-type packaging efficiency*

To more accurately define the packaging capacity of the AAV virion another series of large, genetically wild-type AAV genomes were constructed (see Section 2 details). These large AAV genomes increased sequentially in size by 100 bp. The inserted fragments originated from the Gibco BRL 100 bp DNA size marker, and were ligated into the *Bgl*II site of ins96 via *Bam*HI linkers. The ligated fragments ranged in size from 600 to 1000 bp and this resulted in the cloned AAV plasmids, ins96+0.6, ins96+0.7, ins96+0.8, ins96+0.9, and ins96+1.0 with the insert size indicated within the correspondingly named AAV plasmid. An agarose gel analysis of these plasmids, analyzed by *Pst*I digestion, is shown in Fig. 3. As shown in Fig. 1, the smallest *Pst*I fragment (~0.45 kb) encompasses the right AAV terminal region of AAV with the *Bgl*II restriction site at nt 4484 (map unit 96). This fragment increased in size in constructs ins96+0.5 to ins96+1.0, corresponding to the size of the 600–1000 bp fragment which was ligated into it (Fig. 3).

The series of stepped 100 bp ins96 genomes were then analyzed in the sequential 2 plate assay for virus production. Both HeLa and SW13 cells were used in this analysis. As shown in the top (first plate) of Fig. 4, using Hela cells and using the 100 bp DNA ladder DNA as ^{32}P-labeled probe, all of the large ins96 genomes were able to replicate at comparable levels. As shown in the top (first plate) of Fig. 5, using SW13 cells and using total AAV DNA as ^{32}P-labeled probe, all of the large ins96 genomes were able to replicate at comparable levels to unaltered wild-type AAV, pSM620. In Fig. 6, to better observe the size differences of the AAV genomes the replicating DNAs from the same Hirt DNAs of Fig. 3 were further analyzed by Southern blot analysis after *Pst*I digestion (^{32}P-labeled 100 bp ladder marker DNA as probe). As can be seen the there is a corresponding, ascending increase in size of the replicated DNA comparable to increase in size of the inserted DNA. No smaller, deleted, *Pst*I fragments are observed below the full-length bands indicating that significant deletions of the 100 bp marker DNA inserts are not taking place.

In contrast to near equal replicating abilities in the first plate, differences became apparent for one mutant, ins96+1.0, in its ability to produce infectious virus. As shown in the bottom of Figs. 4 and 5 (bottom, second plate), the large AAV genomes ins96+0.6, ins96+0.7, ins95+0.8, and

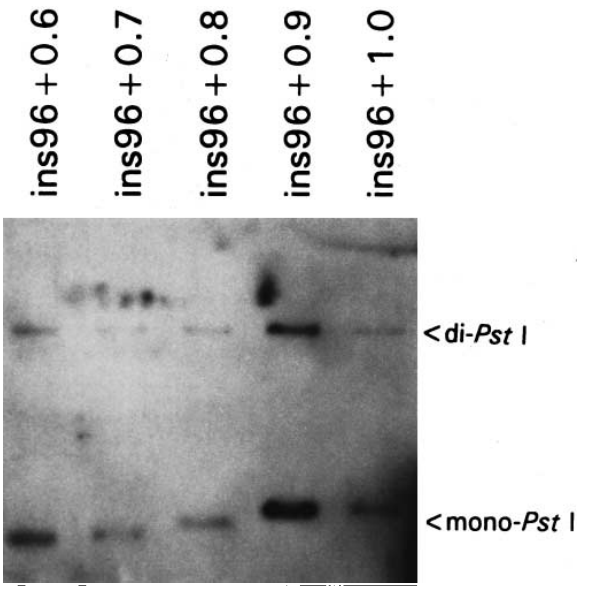

Fig. 6. Size analysis of the replicating DNA of the large AAV genomes. Shown is a *Pst*I restriction analysis of replicating (Hirt) DNA from the first plate of Fig. 4. After digestion the DNA was Southern blotted and probed with ^{32}P-labeled 100 bp ladder DNA. With this probe only the altered right *Pst*I fragment is viewed. Indicated are the monomer and dimer (from tail-to-tail dimers) size left *Pst*I fragments which contain the inserts. Note that the *Pst*I fragments increase in size corresponding to the size of the insert, with ins96+0.6 giving the smallest, and ins96+1.0 giving the largest sized products. Also note that deleted fragments are not visible. This indicates that recombination and elimination of the 100 bp repeats is not taking place at significant levels.

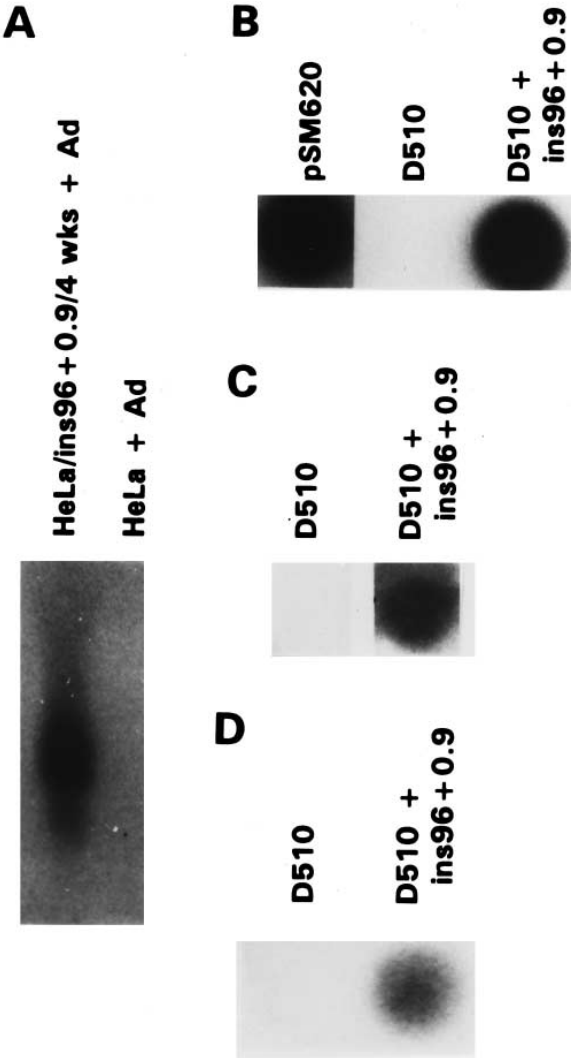

Fig. 7. Latent infection and chromosome 19 integration the ins96+0.9 virus genome. A: Southern blot analysis of Ad2 rescue of HeLa cells latently infected with ins96+0.9 virus 4 weeks previous. B: PCR amplification/dot-blot analysis for AAV *rep* DNA sequences from D510 cells latently infected with ins96+0.9 virus 4 weeks previous. C: PCR amplification/dot-blot analysis for AAV–chromosome 19 junction DNA sequences from D510 cells latently infected with ins96+0.9 virus 4 weeks previous. In more detail: A: Southern blot demonstrating the rescue of ins96+0.9 from latently infected HeLa cells. One thousand HeLa cells were infected with 10^6 infectious units of ins96+0.9 virus. The cells were grown and passed for 4 weeks upon which time the cells were infected with Ad2 and 48 h later a Hirt DNA extraction was carried out. One-third of the extracted DNA was agarose gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled 100 bp marker DNA. Note that a significant level of replicating ins96+0.9 DNA is observed indicating a significant level of latent infection by this virus. B: D510 cells were latently infected with ins96+0.9 virus as in (A). Total cellular DNA was isolated. One microgram of the cellular DNA was used for PCR amplification with primers designed to amplify part of the AAV *rep* gene (24). One-tenth of the PCR product was then dot-blotted and probed using a sequence located between the two primers. Note that a significant signal is present in the D510+ins96+0.9 well. C: Demonstration of ins96+0.9 specific integration into chromosome 19 S1 sequences by PCR amplification of the AAV TR–human chromosome 19 junction sequences probed with an internal AAV TR sequence. One microgram of the cellular DNA from (B) was used for PCR amplification with primers designed to amplify the AAV–chromosome 19 junction within the prefered site of AAV integration (9). One-tenth of the PCR product was then dot-blotted and probed using an AAV TR sequence located between the primers. Note that a significant signal is present in the D510+ins96+0.9 well indicating chromosome 19 integration. D: Demonstration of ins96+0.9 specific integration into chromosome 19 S1 sequences by PCR amplification of the AAV TR–human chromosome 19 junction sequences probed with an internal chromosome 19 sequence. This experiment was carried out similar to (C), except that the dot-blot was probed with a chromosome 19 sequence 5′, internal, of the chromosome 19 junction primer. Note that a significant signal is present in the D510+ins96+0.9 well indicating chromosome 19 integration.
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ins96+0.9 were able to effectively produce virus as judged by their DNA replication in the second plate. In contrast, ins96+1.0 was significantly lower in virus production, in both HeLa and SW13 cells. In three experiments ins96+0.9 was at 70.5 ± 32%, and ins96+1.0 at 25.7 ± 16.2%, the level of pSM620 in the second plate as determined by densitometric analysis. Ins96-λ-M was again shown to be very inefficient in generating infectious virus (as in Fig. 2). However, again, upon very long exposure it was observed that ins96-λ-M did often produce low levels of infectious virus (estimated to be ~0.1–1% that of wild type). From these data it was determined that genomes 0.9 kb larger than wild type were able to be packaged into infectious AAV virus particles at levels only slightly depressed compared to wild type.

3.3. Infectious ins96+0.9 kb virus are able to latently infect cells by chromosome 19 S1 integration

We next assessed if these large AAV genomes were able to latently infect cells by chromosomal integration as does fully wild-type AAV. To determine if this was the case 10^3 HeLa cells were infected with ins96+0.9 virus stock. The cells were grown to confluence, then passed three times after 1:10 splits. After 4 weeks of continuous growth the HeLa/ins96+0.9 cells were then infected with adenovirus to rescue any chromosomally integrated ins96+0.9 genomes. The Southern blot analysis of Hirt DNA using ^{32}P-labeled 100 bp marker probe of Hirt DNA from these cells is shown in Fig. 7A. A significant level of replicating ins96+0.9 DNA is seen. Although these data do not directly demonstrate that the ins96+0.9 genome is chromosomally integrated, after 4 weeks of cell growth it is likely that no significant level of episomal AAV DNA would survive out to this time. The demonstration of significant ins96+0.9 replicating DNA after Ad2 infection suggests that considerable levels of chromosomal integration have taken place.

To demonstrate that the ins96+0.9 virus was specifically integrated into human chromosome 19, 10^3 D510 cells were infected with ins96+0.9 virus stock. The cells were grown to confluence, then passed 3 times after 1:10 splits. After over 4 weeks of continuous growth genomic DNA was isolated and analyzed by PCR amplification/dot-blot hybridization by two procedures. First, shown in Fig. 7B, PCR amplification and hybridization for AAV *rep* sequences were carried out. The D510 cells latently infected with ins96+0.9 virus gave a very strong signal. These data are consistent with significant AAV being present, most likely integrated. However these data do not conclusively indicate specific chromosome 19 S1 integration. As a selectable marker gene is not present within ins96+0.9 it was not possible to observe chromosomal integration by Southern blot analysis of genomic DNA. Next, to

demonstrate chromosome 19 S1-specific integration, PCR amplification of the AAV TR–chromosome 19 junction sequences was carried out, as shown in Fig. 7C,D. The chromosome 19 S1-side primer is of sequences located at the preferred site of AAV integration [10]. The AAV–chromosome 19 junction PCR amplification product was identified by two different probes in dot hybridization assays. One ^{32}P-labeled probe, used in Fig. 7C, was of an AAV TR sequence which was internal to the upstream AAV TR amplification primer. Another ^{32}P-labeled probe, used in Fig. 7D, was of a chromosome 19 sequence which was internal to the chromosome 19 amplification primer. Upon using both probes, the PCR amplified DNA from the D510 cells latently infected with ins96+0.9 virus gave a positive signal (Fig. 7C,D). As this PCR amplification is based on one primer of AAV TR sequences and the other of human chromosome 19 S1 sequences, these data unambiguously indicate a direct, covalent linkage between the AAV and chromosome 19 S1 sequences.

4. Discussion

In this study we demonstrate that the AAV virus particle is able to accommodate 900 additional nucleotides above wild-type size (119%) with reasonable efficiency. This size represents a maximum limit for the construction of recombinant AAV vectors. Furthermore, this study raises the feasibility of constructing and using *wild-type-plus* AAV vectors for gene therapy purposes. Genes of 900 bases or less can be inserted into AAV and still allow the resulting vector to have a largely wild-type phenotype. For example, several antisense genes could be inserted and still allow packaging. In preliminary data we have inserted the Neomycin resistance gene (*Neo*) coding sequence into AAV at map unit 96. Recombinant ins96-0.9Neo virus are able transduce HeLa cells and confer G418 resistance even though the *Neo* gene does not have a dedicated promoter (Hermonat and Han, unpublished). Such *wild-type* vectors would have several advantages or disadvantages. First, high titers of such vectors should be easy to accomplish. Second, the vectors, since they include the *Rep78* gene, would integrate specifically into human chromosome 19. Third, such vectors, being phenotypically *wild type*, have a potential to spread after their initial introduction. The ability for such secondary spreading could be considered an advantage or disadvantage depending upon the purpose and possible activity of the inserted foreign gene. Generally, the Food and Drug Administration (FDA) and some others consider the possibility of such continuous spreading to be unacceptable. However, such spreading may be desirable in certain treatment situations such as in metastatic cancer and AIDS. In any case, secondary spreading would likely be limited, due to anti-AAV antibodies, in all but the most severely immune-compromised individuals. Furthermore, the large *wild-type* vectors will be at a packaging disadvantage when cells are co-infected with true wild-type AAV or with large *wild-type-plus* vectors with deletion mutations within the foreign gene (wild-type D.I.s). As AAV has not been demonstrated to be the etiologic agent of any disease, the FDA may ultimately allow the clinical use of such *wild-type* AAV vectors.

Although this study directly addresses the packaging capacity of the AAV virion, the large genomes which we have used may not represent the packaging efficiency of all recombinant AAV vectors. Equivalently large 'gutted' recombinant AAV genomes, based on only the AAV TRs may have a slightly different packaging capacity. Such large minimal AAV vectors may not to be packaged as efficiently as the vectors of this study as wild-type AAV is preferentially packaged over recombinant AAV of equal size (Hermonat, unpublished). This difference may be due to the elimination of DNA sequences (e.g. secondary structure or 'minor' packaging sequences) located between the AAV terminal repeats (TR). Furthermore, interaction, or lack of interaction, of the inserted foreign DNA with the AAV encoded Rep78 protein may also alter the efficiency of packaging. The AAV encoded Rep78 protein been shown to interact both with the AAV capsid proteins [23] and AAV TR DNA [24], as well as multiple sequences within human chromosomal DNA [19,25]. Thus, the specific foreign DNA which is inserted into the AAV vector will likely affect packaging, due to differing interactions with the packaging machinery. Extending this idea further, it may be that every foreign gene inserted into AAV may result in a vector with a somewhat different packaging capacity.

Another possible use of such large *wild-type-plus* AAV genomes is as complementors for fully defective AAV vectors. Such complementors can be delivered by virus infection and, thus, be easily introduced into 100% of the cells used to produce virus. Virus infection is likely be superior to synthetic techniques for introducing DNA into tissue culture producer cells. When large *wild-type* AAV complementor virus are used in conjunction with AAV vector virus, allowing for the introduction of both into all cells, this technique is likely to be a very good system, if not the best, for producing the highest titers of recombinant AAV virus particles without concentration.

Acknowledgements: This study was funded by a grant from Research to Prevent Blindness and the Arkansas Science and Technology Authority to P.L.H.

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