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Cloning of adeno-associated virus into pBR322: Rescue of intact virus from the recombinant plasmid in human cells

(latent viruses/excision/cruciforms/palindromes)

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ABSTRACT **We have cloned intact duplex adeno-associated virus (AAV) DNA into the bacterial plasmid pBR322. The AAV genome could be rescued from the recombinant plasmid by transfection of the plasmid DNA into human cells with adenovirus 5 as helper. The efficiency of rescue from the plasmid was sufficiently high to produce yields of AAV DNA comparable to those observed after transfection with equal amounts of purified virion DNA. Thus, the recombinant plasmid itself may be a model for studying the rescue of a latent AAV viral infection. In addition, the efficient rescue of viable AAV from the recombinant plasmid should facilitate the genetic analysis of AAV. Finally, the results of an analysis of the DNA from rescued virions indicate that an inversion of the AAV terminal sequences occurred during replication.**

Adeno-associated virus (AAV) is a defective parvovirus that requires helper virus co-infection for its own replication (for review, see ref. 1). In the absence of helper virus, AAV DNA can integrate into the host genome to establish a latent infection (2). The junction between the cellular and viral sequences occurs at the termini of the viral genome but does not seem to involve a specific site in the host genome (ref. 2; unpublished data). In the case of latently infected human cells, AAV may be rescued with high efficiency (up to 10% of latent cells yield virus) upon challenge with either adenovirus or herpes simplex virus (3).

The AAV genome is a linear single-stranded DNA molecule of M_r 1.5×10^6. Strands of both polarities are encapsidated with equal frequency in separate virions (4). AAV-2 DNA has an inverted terminal nucleotide sequence repetition of 145 nucleotides (5). The first 125 nucleotides form a palindromic sequence that can fold back on itself to form a "T"-shaped hairpin structure and can exist in either of two orientations (flip or flop) in virion DNA. This has led to the suggestion (1) that AAV DNA replicates according to a model first proposed by Cavalier-Smith (6) in which the terminal hairpin of AAV is used as a primer for the initiation of DNA replication. This model is illustrated in Fig. 1 and has been supported by studies of *in vivo* AAV DNA replication (1).

Because AAV cannot form plaques in the absence of helper virus, the genetic study of AAV has been difficult. In this paper we report the cloning of the intact AAV genome into the bacterial plasmid pBR322 and the ability of the AAV genome to be rescued from the recombinant plasmid upon transfection into human cells. The rescued AAV genome is fully functional in that it is replicated and infectious virus are produced that are indistinguishable from wild-type virions.

METHODS

Cell Culture and DNA Transfection. 293-31 cells (7), a human line transformed with adenovirus type 5 (Ad5), were maintained in modified Eagle's medium with Hanks' salts (GIBCO), penicillin, streptomycin, and 10% fetal calf serum. Cells were transfected by using the DEAE-dextran method (8) as described (9) except that the DNA concentration was 0.2–10 μg of superhelical closed circular (form I) plasmid DNA or linear duplex AAV DNA per ml per 10-cm dish. The transfecting solution also contained Ad5 virus [multiplicity of infection (moi) = 10]. Plaque-purified Ad5 was obtained from Peter McGuire. In some experiments, cells were labeled with $^{32}P_i$ (200 μCi/10-cm dish; 1 Ci = 3.7×10^{10} becquerels) between 16 and 48 hr after transfection (9).

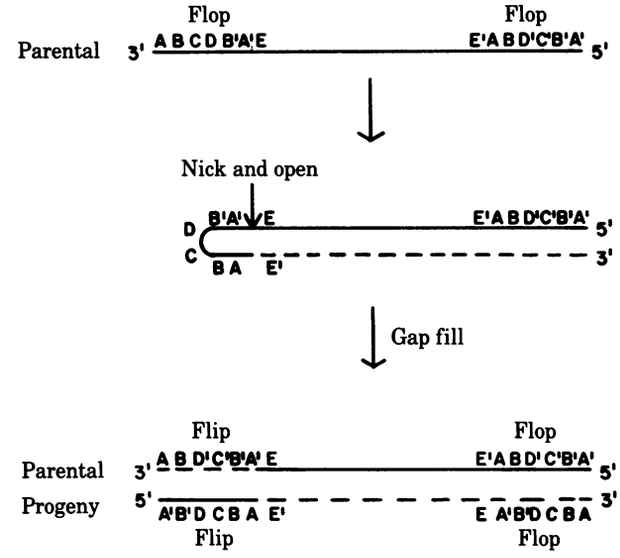

FIG. 1. Model for AAV DNA replication. C and D represent two different palindromic sequences which are themselves within a larger palindrome, AB. . .B'A'. DNA synthesis proceeds from the 3'-terminal hairpin to the end of the molecule, essentially forming a "shortened" DNA molecule. A putative site-specific enzyme makes a single nick on the parental strand opposite the original 3' end of the parental strand. This results in inversion of the terminal repeat and transfer of the parental palindromic sequence to the daughter strand. The parental DNA is then extended to give the full-length duplex genome. Because duplex AAV DNA is the product of *in vitro* hybridization and because any specific DNA strand can have either orientation (flip or flop) at either end, 75% of the molecules will have mismatched sequences at (at least) one end.

DNA Extraction and Hybridization Procedures. Plasmid DNA and AAV DNA were isolated as described (9, 10). Low molecular weight DNA was isolated from 293-31 cells after DNA transfection by the method of Hirt (11) as described (9). The DNA was then fractionated by electrophoresis on 1.4% agarose gels, transferred to nitrocellulose (12), and hybridized

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Abbreviations: AAV, adeno-associated virus; bp, base pair(s); Ad5, adenovirus 5; form I, superhelical closed circular DNA; form II, nicked circular DNA; form III, linear DNA; moi, multiplicity of infection.

to ^{32}P-labeled nick-translated DNA ($0.8–2 \times 10^8$ cpm/μg) as described (9).

Enzymes were obtained from Bethesda Research Laboratories and used as suggested by the supplier unless otherwise indicated. Restriction fragments were extracted from acrylamide or agarose gels (13), filtered (0.45-μm Millipore filter), extracted with phenol, dialyzed against water, and concentrated by evaporation.

Hae III-digested fragments were labeled with ^{32}P at their 5′ ends by the use of bacterial alkaline phosphatase and T4 polynucleotide kinase (13). *Bgl* I-digested fragments were labeled with ^{32}P at their 3′ ends by using the Klenow fragment of *Escherichia coli* polymerase I. Approximately 0.1 μg of DNA was incubated for 5 min at 25°C with 1 unit of enzyme in 0.05 ml containing 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 μCi of [α-^{32}P]dCTP (0.8 Ci/μmol), and unlabeled dGTP, dTTP, and dATP at 1 mM each. The DNA was then precipitated and washed with ethanol and subjected to electrophoresis. Because of the heterogeneity of the *Bgl* I site and because of the fact that all four deoxynucleoside triphosphates were present in the Klenow reaction mixture, the *Bgl* I ends were not uniformly labeled by this procedure. Nevertheless, comparison of the *Bgl* I digestion pattern with patterns obtained when only [α-^{32}P]dCTP was used indicates that all of the *Bgl* I fragments were labeled.

Cloning Procedures. pBR322 and HB101 (14) were obtained from Hamilton Smith. AAV duplex linear DNA and pBR322 linear DNA, which had been cleaved with *Pst* I, were treated with terminal deoxynucleotidyl transferase (15) in the presence of [α-^{32}P]dCTP (3.2×10^8 cpm/μmol) and [α-^{32}P]dGTP (5.3×10^8 cpm/μmol), respectively. Approximately 30 dGMP residues were added to each end of the pBR322 DNA and 8 residues of dCMP were added to each end of the AAV DNA. The tailed DNA species were separated from unreacted deoxynucleoside triphosphates by electrophoresis on a 1.4% agarose gel and then hybridized to each other as described (16). The hybridized DNA was then transfected into HB101 as described (10). Colonies that were resistant to tetracycline and sensitive to ampicillin were isolated (14) and screened by colony hybridization (17) for AAV DNA sequences. All work with recombinant plasmids was done in accordance with the National Institutes of Health guidelines for recombinant DNA experiments.

RESULTS

Cloning of Intact AAV DNA into pBR322. Terminal deoxynucleotidyl transferase was used to attach oligo(dG) to the 3′ termini of pBR322 *Pst* I linear molecules and oligo(dC) to duplex AAV DNA. The modified pBR322 and AAV DNA molecules were then hybridized and the recombinant molecules were transfected into *E. coli* HB101. The frequency of transformation was found to be only 9% of the frequency obtained in a similar experiment in which simian virus 40 DNA was cloned into pBR322. This was the first of several difficulties encountered in the cloning of AAV (see *Discussion*).

A total of 50 colonies were obtained which contained AAV DNA sequences. Only three of these contained plasmids that were large enough to be approximately the correct size for an intact AAV recombinant plasmid. These three plasmids (pSM501, -503, and -504) were subjected to restriction enzyme analysis with *Pst* I, *Bam*HI, and *Sma* I. Digestion with *Pst* I (Fig. 2 *A* and *C*) indicated that there were no major deletions in the nonrepeated internal sequences of AAV and that the *Pst* I site of pBR322 was re-formed during the cloning procedure. (Only pSM501 and 504 are shown in Fig. 2*A*.) This was confirmed by digestion with other restriction enzymes (*Hin*cII, *Bal* I) as well as by hybridization of restriction digests with ^{32}P-labeled probes specific for internal AAV fragments (data not shown). In all three clones, the internal AAV sequences between 0.10 and 0.90 map units appeared to be intact.

Digestion with *Bam*HI, however, demonstrated that all three clones contained mixtures of at least two plasmids which differed from each other by virtue of deletions in the terminal sequences of AAV (data not shown). To see if any of the clones contained AAV DNA with unaltered terminal repeated sequences the DNAs were digested with *Sma* I. *Sma* I cleaves only within the terminal repetition of AAV DNA at either nucleotides 45 and 57 or 68 and 79, depending on the orientation of the terminal sequence. Thus, a recombinant plasmid containing intact AAV DNA would be cut at both ends of the AAV insert to yield two linear products of approximately equal length. Two of the three clones (pSM501 and pSM504) contained some plasmids that were cut at the expected sites (pSM501 is shown in Fig. 2*B*, lane 3).

In order to obtain clones that contained single plasmid species, the plasmid DNA from pSM501 was isolated and retransfected into *E. coli* HB101 at low concentration. Two of the subclones obtained, pSM620 and -621, were highly enriched for a single plasmid species which represented one or the other of the major species contained in pSM501 (Fig. 2*B*). Of these, pSM620 contained the recombinant plasmid that was cut at both termini of the AAV insert by *Sma* I. Studies with other restriction enzymes (not shown) indicated that the small amount of linear (form III) DNA in Fig. 2*B* (lane 1) was not due to incomplete digestion with *Sma* I but rather to the presence of a variant

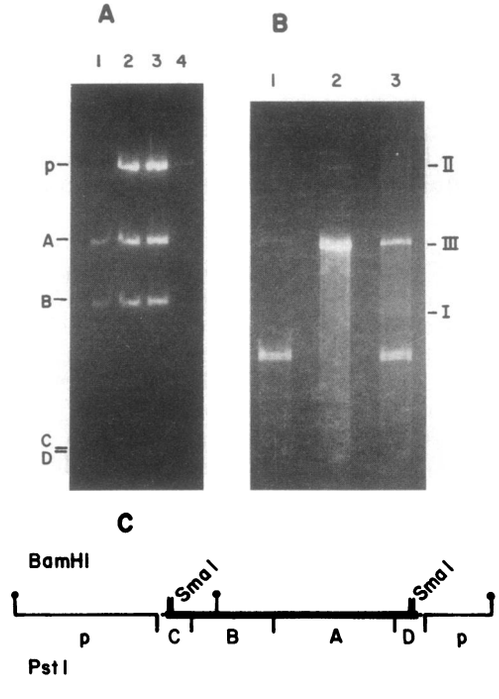

FIG. 2. (*A*) *Pst* I digestion fragments of AAV (lane 1), pSM501 (lane 2), pSM504 (lane 3), and pBR322 (lane 4) DNA. The digested DNA was fractionated on a 1.4% agarose gel, and the gel was stained with ethidium bromide. p, pBR322 linear plasmid DNA; A–D, AAV *Pst* I fragments A–D. The AAV terminal *Pst* I fragments (C and D) are poorly resolved on this type of gel. (*B*) *Sma* I digestion fragments of subclones pSM620 (lane 1) and pSM621 (lane 2) and parental clone pSM501 (lane 3). I, II, III, form I, form II, and form III of the recombinant plasmid DNA. Electrophoresis was as in *A*. (*C*) Restriction enzyme map of pSM620 drawn as if the plasmid were cleaved at the *Bam*HI site in pBR322. The interruption in the line (dot) indicates the position of the G-C tail. p and A–D, *Pst* I fragments as in *A*. The positions of the *Bam*HI sites indicate the orientation of AAV sequences with respect to pBR322. The *Sma* I sites within the AAV terminal repetition are also indicated.

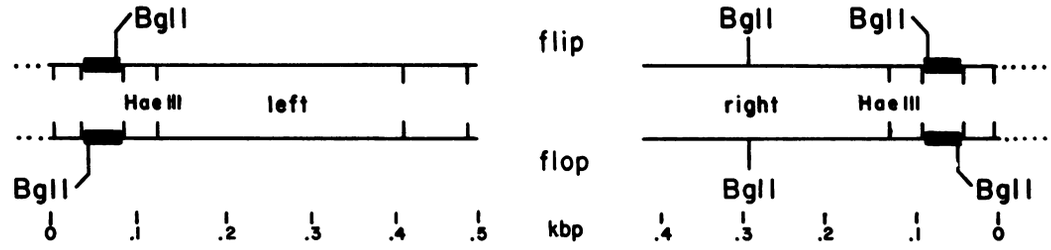

FIG. 3. *Hae* III and *Bgl* I restriction maps of the AAV terminal *Pst* I fragment C (left end) and fragment D (right end) in either the flip (top lines) or flop (bottom lines) orientation. Whereas wild-type duplex AAV DNA contains all possible combinations of flip and flop at the two ends, pSM620 has only the flop orientation at both ends. Solid boxes, region affected by the inversion. kbp, kilobases.

plasmid (1–5%) in pSM620 with a deletion in one of the AAV termini.

Plasmid DNA from pSM620 was further characterized by digestion of the *Pst* I AAV terminal fragments with *Bgl* I. *Bgl* I cuts *Pst* I fragment D (right end of AAV) at either nucleotide 43 (flop) or 79 (flip), depending on the orientation of the terminal sequence, and at nucleotide 298 (Fig. 3). Thus, three fragments would have been expected, and were in fact observed, from *Pst* I fragment D of AAV (Fig. 4). Two of these fragments [121 and 255 base pairs (bp)] were indistinguishable in size from fragments obtained from wild-type AAV. The presence of the 255-bp fragment indicated that the right-hand AAV terminus in pSM620 was in what we have arbitrarily called the flop orientation. The third fragment was larger than its wild-type AAV counterpart, presumably by virtue of the G·C residues added in the tailing procedure. Based on its mobility, we estimated that the right-hand AAV junction contained approximately 25 G·C residues. (An additional band of approximately 100 bp which was seen in both viral and plasmid DNA was an artifact of the labeling procedure.) From a similar analysis of the left-end AAV terminal *Pst* I fragment (Figs. 3 and 4) we concluded that the left-end AAV terminal repeat was also in the flop orientation and contained approximately 15 G·C residues. Finally, digestion of the *Pst* I terminal fragments with *Hpa* II and *Hae* III (not shown) essentially confirmed this analysis. Thus, to the level of resolution afforded by restriction enzyme digestion, we concluded that the ends of AAV DNA were intact in subclone pSM620. Additionally, we have determined the sequence of the terminal 41 nucleotides of the inserted AAV genome in pSM620. There were no alterations in the sequence at either end. There were 26 G·C residues at the right terminus and 16 G·C residues at the left end, in good agreement with the estimate from restriction digests.

Biological Activity of pSM620 in Human Cells. Plasmid DNA from pSM620 was transfected into 293-31 cells which were then co-infected by Ad5. At 48 hr after infection the cells were extracted by the method of Hirt (11) and the supernatant fluid was fractionated on 1.4% agarose gels and blotted by the Southern procedure (12). Several bands containing AAV sequences were detected by hybridization (Fig. 5*A*, lane 3). All of these were bands that were observed in the course of normal AAV DNA replication (Fig. 5*A*, lane 1). No bands were detected in cells infected by Ad5 alone or in cells that had been transfected with just the plasmid DNA (Fig. 5*A*, lanes 2 and 4). This suggested that AAV DNA had been rescued from the recombinant plasmid and replicated. To assess this possibility further, cells that had been transfected as above were labeled with $^{32}P_i$ for 30 hr prior to the extraction of DNA. Electrophoresis of the Hirt supernatant fluid revealed labeled bands at positions characteristic of replicating AAV DNA (Fig. 5*B*). Again, these bands were absent from the lanes containing the control cells infected by Ad5 alone or transfected by plasmid alone. Thus, we concluded that AAV DNA was rescued from the recombinant plasmid and replicated. Although it is difficult to calculate the efficiency of rescue precisely, the nearly comparable yields of AAV DNA obtained from cells transfected with either pSM620 or linear duplex AAV DNA (Fig. 5*A*) leads us to conclude that the rescue must be quite efficient.

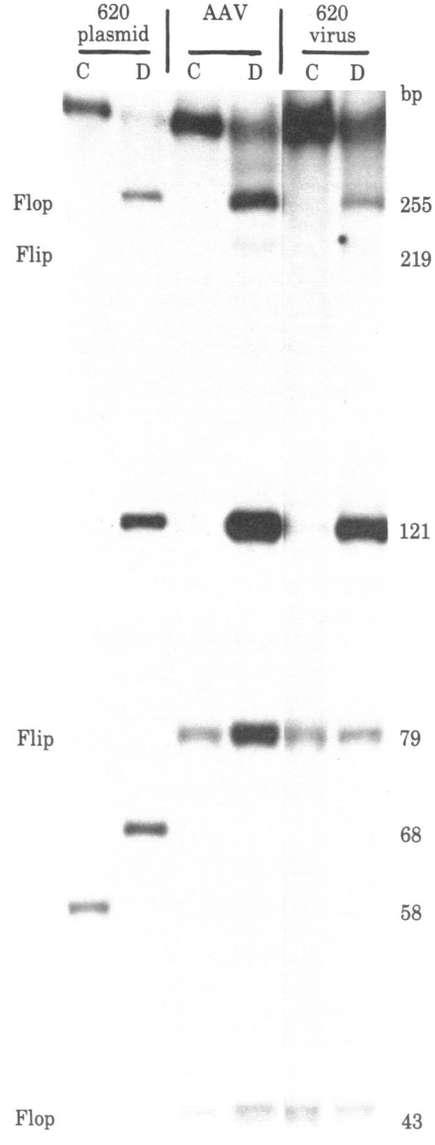

FIG. 4. *Bgl* I digestion of AAV *Pst* I terminal fragments. The AAV *Pst* I fragment C (left end) and *Pst* I fragment D (right end) were isolated from pSM620 plasmid DNA (left two lanes) or from AAV duplex DNA (middle two lanes) or from rescued viral DNA (right lanes) and then digested with *Bgl* I and labeled at their 3′ ends with ^{32}P. The labeled DNA was then fractionated on an 8% acrylamide gel. C and D, *Pst* I fragments C and D; Flop and Flip, fragments generated by these two orientations.

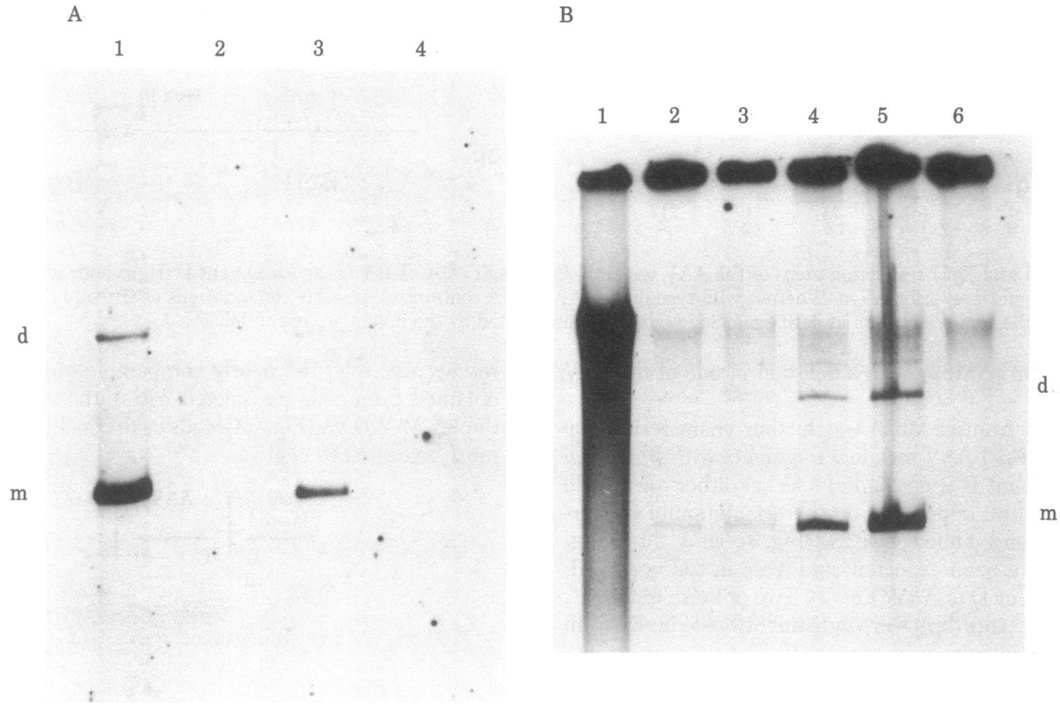

FIG. 5. (*A*) Southern blotting. Low molecular weight DNA was isolated (11) from 293-31 cells that had been infected with AAV DNA and Ad5 (lane 1), Ad5 alone (lane 2), pSM620 DNA and Ad5 (lane 3), and pSM620 DNA alone (lane 4). The DNA was fractionated on a 1.4% agarose gel, transferred to a nitrocellulose filter by the Southern method (12), and hybridized to nick-translated ^{32}P-labeled AAV DNA. (*B*) *In vivo* ^{32}P-labeling. 293-31 cells were infected with pSM620 plasmid DNA alone (lane 1) or with increasing amounts of pSM620 DNA and Ad5 (lanes 2–5) or with Ad5 alone (lane 6). The amount of Ad5 was constant (moi = 10). The amount of pSM620 DNA in lanes 1–5 was 10, 1, 2, 5, and 10 μg, respectively. The infected cells were labeled with $^{32}P_i$ for 30 hr prior to harvesting. Low molecular weight DNA was isolated and fractionated as in *A*. Each lane contains the DNA from one 10-cm dish.

To determine whether infectious virus was produced, transfected cells (one 10-cm dish) were harvested at 48 hr, frozen and thawed twice, and then added without further purification to a HeLa cell suspension culture (2×10^8 cells) together with Ad5 helper (moi = 10). At 48 hr the HeLa cells were harvested and any AAV virions produced were purified by CsCl centrifugation as described (18). Only cell lysates that were the result of infection by both pSM620 plasmid DNA and helper virus produced visible AAV virion bands. Once again, infection with Ad5 alone produced no visible virus band. DNA isolated from the rescued virions gave a band pattern, after *Pst* I digestion, that was indistinguishable from that of normal AAV DNA (Fig. 6).

Finally, because both the AAV termini in the plasmid were in the flop orientation, this was an opportunity to test a specific prediction of the current model for AAV DNA replication. Since the discovery that the terminal sequences of AAV can exist in either of two orientations (flip or flop), an attractive model for AAV replication has been the one first proposed by Cavalier-Smith (see Fig. 1 and ref. 6) and elaborated in detail by Berns and Hauswirth (1). In this model the terminal sequences of AAV are inverted during the course of replication to generate the two possible orientations. A specific prediction of this model is that a parental molecule with a given orientation at both ends (flop, flop) would yield progeny molecules with both flip and flop orientations at either end. This indeed is what we find when we examine the orientation of the AAV termini in the virus that has been rescued from plasmid DNA. When the *Pst* I terminal fragments C and D were isolated from rescued virion DNA and digested with *Bgl* I, both orientations were seen (Fig. 4) in both terminal fragments. Thus, an inversion had occurred at both ends of the AAV genome during the rescue and replication of the AAV sequences from the recombinant plasmid.

DISCUSSION

We have described a recombinant plasmid containing the intact AAV genome in pBR322. The AAV genome can be rescued from the recombinant plasmid by transfection of the plasmid into human cells. We suspect that the ability of the AAV genome to be rescued under these conditions reflects two factors. The first is the major role that latent infection may play in the life cycle of AAV. As a defective virus that requires a helper to undergo lytic infection, AAV has the problem of maintaining the biological continuity of its genetic information until its host is infected by a helper virus. The capability of establishing a latent infection from which the genome can be efficiently rescued by the helper virus circumvents this problem. Second, although AAV DNA integration is specific for the AAV ends, it does not appear to be specific for host DNA sequences. A Southern hybridization analysis of two independently isolated clones of cells latently infected by AAV has indicated that the site for integration into cellular DNA is not unique (ref. 2; unpublished data). If there is no discernible host nucleotide sequence specificity required for rescue, then the recombinant plasmid described in this paper may represent an accurate model for integrated AAV DNA. Thus, the events observed upon transfection would be pertinent for both rescue of the latent AAV genome and its replication.

A particular advantage of the recombinant plasmid described is its potential utility in defining specific functions along the AAV genome. During the isolation of the wild-type AAV recombinant clone pSM620, a number of additional clones were isolated in which there were deletions at the termini of the AAV genome (pSM621 is one example). Of interest is how much the unusual structure formed by the terminal 125 nucleotides in AAV DNA may be altered without loss of function. Finally, we

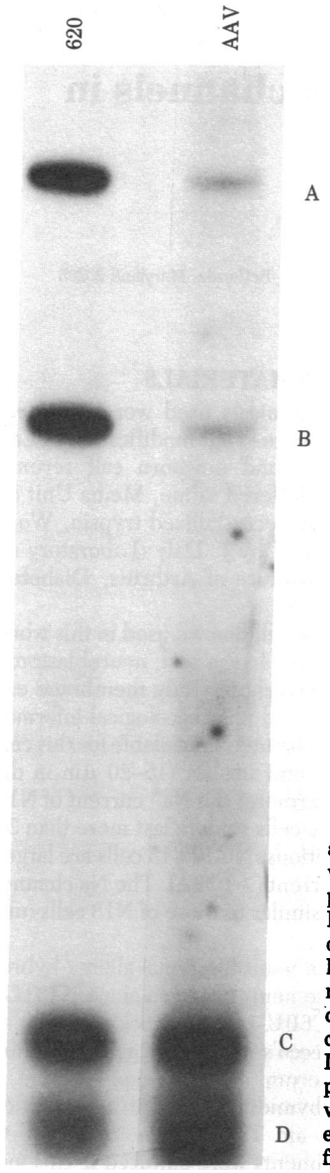

FIG. 6. Wild-type AAV DNA and DNA isolated from rescued virions, after transfection by pSM620, were digested with *Pst* I and fractionated on a 2% agarose gel. The *Pst* I fragments were labeled with the Klenow fragment in the presence of [α-^{32}P]-dCTP. The apparent lack of stoichiometry among the wild-type DNA fragments is due to the presence of defective interfering viral DNA whose sequences are enriched for the *Pst* I terminal fragments.

are now in a position to perform site-specific mutagenesis experiments along the length of the AAV genome, which should enable us to determine the number of complementation groups in AAV and to test the potential utility of the AAV genome as a generalized vector in human cells.

A major concern with the experiments described in this paper is the possibility of contamination of the Ad5 helper virus stock with AAV. There is no detectable contamination by intact AAV as shown by the absence of AAV DNA replication or the production of AAV virions in human cells infected with the Ad5 stock alone. A more remote possibility would be contamination by a defective AAV which could be rescued by complementation or by recombination with AAV sequences in the recombinant plasmid. This seems unlikely for two reasons. First, by virtue of the inability of such defective particles to replicate without AAV help, it is difficult to envisage how they would be maintained in the Ad5 stock. Additionally, blotting at a sensitivity sufficient to detect single-copy DNA failed to reveal any AAV sequences in cells infected by the adenovirus stock alone (Fig. 5A).

As pointed out in *Results*, a number of difficulties were encountered in the cloning of AAV DNA. The first difficulty was that the frequency of transformation of *E. coli* with AAV/pBR322 recombinants was significantly lower than expected. The second difficulty was that 100% of the primary clones examined contained deletions in the AAV sequences. Of those that were examined in detail, the majority were heterogeneous and only two clones also contained intact AAV DNA. Furthermore, when the plasmids with intact AAV sequences were retransfected into *E. coli*, the AAV termini were unstable and generated new deletions at a low frequency.

We attribute these difficulties to the palindromic terminal sequence in AAV DNA. The terminal palindrome in AAV DNA is of the type ABCDB'A' in which two shorter palindromes (C and D) are contained within a larger palindrome. Because the AAV termini can exist in either of two orientations, flip or flop, 75% of intact duplex AAV DNA molecules, which are formed by *in vitro* hybridization of the single-stranded genome, have a 42-bp mismatched sequence at one or both ends. This combination of circumstances can lead to the formation of several different kinds of cruciform structures (1). These structures should exist at a high frequency in the initial transformation experiments (when the recombinant molecules contain a high frequency of mismatched termini) and at a lower frequency during continued growth in *E. coli*. The largest theoretical cruciform in the AAV terminus (5) would contain 59 paired and 7 unpaired bases (distributed among three loops). A number of laboratories have shown that cruciform structures can occur in supercoiled DNA molecules and that they promote the formation of deletions (19). Thus, the AAV recombinant plasmid we describe here may be useful in studying the effect of cruciform structures on the topology of supercoiled molecules as well as their biological activity in prokaryotes.

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