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In Vitro Excision of Adeno-Associated Virus DNA from Recombinant Plasmids: Isolation of an Enzyme Fraction from HeLa Cells That Cleaves DNA at Poly(G) Sequences

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When circular recombinant plasmids containing adeno-associated virus (AAV) DNA sequences are transfected into human cells, the AAV provirus is rescued. Using these circular AAV plasmids as substrates, we isolated an enzyme fraction from HeLa cell nuclear extracts that excises intact AAV DNA in vitro from vector DNA and produces linear DNA products. The recognition signal for the enzyme is a polypurine-polypyrimidine sequence which is at least 9 residues long and rich in G · C base pairs. Such sequences are present in AAV recombinant plasmids as part of the first 15 base pairs of the AAV terminal repeat and in some cases as the result of cloning the AAV genome by G · C tailing. The isolated enzyme fraction does not have significant endonucleolytic activity on single-stranded or double-stranded DNA. Plasmid DNA that is transfected into tissue culture cells is cleaved in vivo to produce a pattern of DNA fragments similar to that seen with purified enzyme in vitro. The activity has been called endo R for rescue, and its behavior suggests that it may have a role in recombination of cellular chromosomes.

Adeno-associated virus (AAV) has a linear single-stranded DNA viral genome (approximately 5 kilobases [kb]) with inverted terminal repeats that are palindromic (3, 50). For a productive viral infection, AAV requires the presence of a coinfecting helper virus; virtually any member of the adenovirus or herpesvirus family can supply helper function (1, 8, 35). In the absence of a helper virus, AAV readily integrates into the host DNA (4, 15, 21) either as a single proviral copy or, more often, as a tandem head-to-tail array of several AAV genomes (11, 26, 34a).

When a latently infected cell is superinfected with a helper virus, the integrated AAV genome is rescued and proceeds through a normal productive infection (21). In many proviral cell lines the yield of replicative intermediates and virus produced by superinfection is identical to that obtained from exogenous AAV infections (26, 34a). This suggested that rescue is a rapid event initiated by specific cleavage within the AAV termini.

Additional evidence that AAV rescue involves a site-specific nuclease comes from the study of recombinant AAV plasmids. When AAV-pBR322 plasmids are transfected into human cells in the presence of adenovirus, free linear duplex AAV DNA, the major replicative intermediate (16, 51), is seen within 24 h (43). Presumably, rescue from the recombinant plasmid occurs by a mechanism similar to that used for proviruses integrated into chromatin (43). Viable terminal deletion mutants, when compared with wild-type plasmids, are partially defective for the formation of replicative intermediates (29, 45), suggesting that sequences at the termini are required for excision.

In principle, the duplex replicative intermediate can be generated from the covalently closed form I plasmid DNA in one of two ways. Either the AAV sequences are separated from the plasmid by precise excision or a single-stranded DNA molecule is generated by AAV-specific replication (see references 45 and 48 for examples of the second mechanism). Because the AAV termini are also the origins for AAV DNA replication (16, 45), it is difficult to distinguish between the two mechanisms. Both mechanisms, however, require either a specific nick or a double-stranded cut at an AAV-vector junction in the input plasmid DNA. With this in mind, we have used the recombinant plasmids as substrates to isolate an enzyme fraction from HeLa cells that was capable of excising AAV DNA from the plasmids in vitro. We also demonstrated that AAV sequences can be specifically cleaved in vivo in the absence of AAV DNA replication and that the cleavage pattern in vivo is similar to that seen in vitro with partially purified extracts. We have called the enzyme responsible for this rescue activity endo R.

MATERIALS AND METHODS

Cell culture and viruses. HeLa S3 cells were maintained at 37°C in suspension culture in Eagle minimal essential medium supplemented with 5% calf serum, 1% glutamine, penicillin, and streptomycin. Wild-type adenovirus type 2 was prepared from a freeze-thaw lysate of HeLa S3 cells as previously described (45). HeLa monolayer cells were transfected with 5.0 μg of DNA by the DEAE-dextran method (34) as described previously (37) and infected with adenovirus type 2 at a multiplicity of infection of 10. Low-molecular-weight DNA was isolated from the cells by the method of Hirt (19) as described previously (37).

Recombinant clones. All recombinant clones were maintained in either the *recA* host HB101 (7) or the *recBC recF sbcB* host JC8111 (5), a gift from Peter Tattersall. This was to prevent variation in the size of the G · C tails (17) and deletion of the palindromic AAV terminal repeat (43).

The plasmids pGM620C and pGM620D contain either the left or the right terminal *Pst*I fragment, respectively, of the wild-type AAV plasmid pSM620 (45), subcloned into the *Pst*I site of pBR322 (6). Both clones contain the original poly(G)-poly(C) tails that are present in pSM620. Plasmid pGM1008 was subcloned from pGM620D and contains the poly(G)-poly(C) tail plus 21 base pairs (bp) of the terminal

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AAV sequence up to the *Bss*HII site at nucleotide 4657. The AAV/pBR junctions of these clones were confirmed by DNA sequencing.

The plasmids pGMGC9, pGMGC13, and pGMAAVter were constructed by chemically synthesizing oligonucleotides (Systec) containing the sequences C_9-G_9 (pGMGC9), C_{13}-G_{13} (pGMGC13), or GGCCACTCCCTCTCTGCGCGC-GCGCGCAGAGAGGGAGTGGCC (pGMAAVter). In each case both strands of the oligonucleotides were synthesized, so that the *Pst*I recognition site was included. The two strands of each oligonucleotide were annealed and ligated into the *Pst*I site of pBR322. The inserts and pBR322 flanking sequences of these plasmids were also confirmed by DNA sequencing.

The plasmids pGA38 and pAV1 were gifts from Todd Evans and Catherine Laughlin, respectively. pGA38 contains an insert of the alternating copolymer $(G\text{-}A)_{38}$ cloned into the *Eco*RI site of pUC9 (13). pAV1 is an infectious wild-type AAV clone constructed with the use of *Bgl*II linkers into a pBR322 derivative (28). The junctions of pAV1 have not been sequenced.

pSM609, pSM620, pSM621, pSM703, and pSM704 were previously described (45). All of the junctions in these plasmids have been sequenced (31, 46) as part of this study, with the exception of pSM704 (right).

Purification of endo R. All operations were carried out at 0 to 4°C. Nuclei were isolated from HeLa S3 cells essentially as described by Challberg and Kelly (10). Frozen nuclei were thawed on ice and extracted for 2 h with buffer A (25 mM Tris hydrochloride [pH 8.0], 10 mM KCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing 0.2 M NaCl. The nuclei were then removed by centrifugation (3,000 × *g* for 15 min), and the supernatant was dialyzed against buffer A. Any insoluble precipitate in the nuclear extract was removed by centrifugation at 12,000 × *g* for 20 min, and the supernatant was retained as fraction I.

Fraction I was loaded onto a 16-ml DEAE-cellulose column equilibrated with buffer A, and endo R activity was eluted with a 150-ml linear gradient of buffer A containing KCl (0.01 to 0.5 M). Active fractions were pooled (fraction II), dialyzed against buffer B (25 mM Bis Tris [Bis Tris is 2,2-bis(hydroxymethyl)-2,2′,2″,-nitrilotriethanol] [pH 6.5], 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing 0.01 M KCl, and loaded onto a 15-ml phosphocellulose column prewashed with 50 ml of the same buffer. The column was eluted with a 150-ml linear gradient from 0.01 to 0.5 M KCl in buffer B. Active fractions were pooled (fraction III) and concentrated about 55-fold by Amicon Centriflo 25 centrifugation. Half of fraction III was then chromatographed on a 36-ml column of Sephadex G-200 (1.5 by 45 cm) equilibrated with buffer A (pH 7.5) containing 25 mM KCl, and active fractions were pooled (fraction IV). Fractions I to IV were stored at −20°C and were stable for at least 3 months without measurable loss of activity.

Nuclease assays. The standard endo R reaction mixtures of 25 μl contained 20 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 pmol of form I plasmid substrate, and 0.1 to 5.0 U of endo R (see below). After incubation at 37°C, the reaction products were phenol extracted, ethanol precipitated, and digested with either *Bst*EIII at 60°C for 1 h or *Sph*I at 37°C for 3 h. The reaction products were then fractionated by electrophoresis on 1.4% agarose gels. One unit of endo R activity is defined as the amount of protein required to cleave 50% of pGM620D substrate under standard conditions. During the early stages of the purification, it was necessary to treat the reaction products with protease (pronase; Sigma Chemical Co.) prior to phenol extraction and gel electrophoresis.

Assay mixtures for single-stranded nuclease contained 5.0 μg of heat-denatured ^{3}H-labeled *Escherichia coli* chromosomal DNA (10^5 cpm/μg) in 250 μl. For S1, the mixtures contained 50 mM sodium acetate (pH 4.5), 0.3 M NaCl, 10 mM $ZnCl_2$, and 1.25 U of S1. The endo R reaction was carried out under standard reaction conditions. Portions (25 μl) were removed at the indicated times and added to 2 ml of 10% trichloroacetic acid containing 200 μg of bovine serum albumin per ml. The precipitates were collected on nitrocellulose filters (BA85; Schleicher & Schuell, Inc.), washed with trichloroacetic acid and ethanol, and counted. *E. coli* DNA was labeled with [^{3}H]thymidine (6.7 Ci/mmol; ICN Pharmaceuticals Inc.) as described previously (22).

Estimate of in vivo excision frequency. A typical transfection uses 0.1 to 10 μg of plasmid DNA per 10-cm dish or approximately 3×10^9 to 3×10^{11} molecules per dish. Assuming that there are 10^6 competent cells per dish, each competent cell will have 3×10^3 to 3×10^5 molecules of plasmid DNA. This agrees reasonably well with the amount of plasmid DNA recovered from transfected cells as measured by filter hybridization. If the in vitro frequency of cleavage by endo R at an outboard site of AAV is 1%, the cleavage frequency at both outboard sites to produce an intact replicative form would be 0.01%. This would produce 0.3 to 30 molecules of excised AAV DNA per transfected cell.

RESULTS

Detection of site-specific nuclease activity in crude nuclear extracts. The primary substrate for the in vitro endo R assay was the plasmid pSM620. This plasmid consists of the wild-type AAV sequence cloned into the *Pst*I site of pBR322 by G · C tailing (43). Form I supercoiled DNA was first treated with cell extract and then digested with a one-cut restriction enzyme that cleaves either in the AAV sequences (*Bst*EII) or in vector DNA (*Sph*I). The products of the assay were then fractionated by agarose gel electrophoresis. Figure 1 illustrates the fragments that would be produced if endo R cleaves at either the left or right AAV-vector junction. For example, endo R cleavage at the right AAV terminal repeat would generate a linear plasmid DNA molecule which, after *Bst*EIII digestion, would produce a 3.0-kb fragment which consists exclusively of AAV DNA and a reciprocal 6.1-kb band that contains both AAV and pBR sequences.

Crude extracts prepared from HeLa cells were assayed for the ability to cut near the AAV-vector junctions, and the products were visualized by transfer to nitrocellulose filters and hybridization to AAV-specific probe. Endo R activity was found in whole-cell extracts and extracts prepared by treating nuclei with 0.2 M NaCl (10), which usually contained fewer contaminating nucleases. In addition, the activity was found to be elevated three- to fivefold in extracts prepared from cells that had been infected with adenovirus type 2 and treated with hydroxyurea from the onset of infection (results not shown). For these reasons, nuclear extracts from adenovirus type 2-infected HeLa cells treated with hydroxyurea were used as a source of the activity.

When crude nuclear extracts were assayed, only 1% of the starting substrate was cleaved at either AAV-vector junction, and the products could be visualized only by Southern

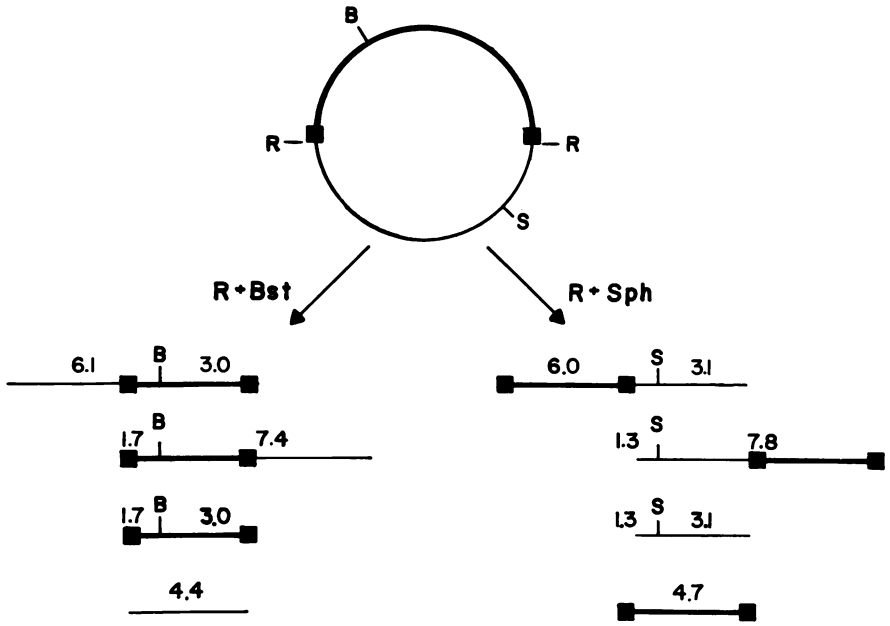

FIG. 1. Endo R cleavage products. The figure illustrates the products that would result from partial digestion by endo R and complete digestion by *Bst*EII (left pathway) or *Sph*I (right pathway) when the wild-type plasmid, pSM620, is used as substrate. ▬, AAV DNA; ——, pBR322 DNA; |, cleavage sites of *Bst*EII (B), *Sph*I (S), and endo R (R); ■, AAV terminal repeats.

hybridization (49). To study the reaction further, we partially purified the enzyme activity by chromatography on DEAE-cellulose and phosphocellulose and by gel filtration (Materials and Methods). The phosphocellulose (fraction III) and gel filtration (fraction IV) fractions were approximately 100- and 500-fold more pure than the crude nuclear extract was and were essentially indistinguishable in our assays. A complete account of the purification of endo R will be published elsewhere (Gottlieb and Muzyczka, in preparation).

Products of endo R reaction. Figure 2 illustrates an enzyme titration of the fraction III enzyme with the plasmid pSM620 as substrate. As the reaction goes to completion, most of the AAV-vector junctions are cleaved to produce the AAV 1.7- and 3.1-kb fragments expected from cleavage with *Bst*EII and endo R (compare with Fig. 1). At high concentrations of enzyme, minor cleavage fragments are also seen. The most prominent of these are 2.4- and 1.6-kb fragments. The sizes of these fragments suggest that their recognition sequences must also occur within AAV DNA.

To demonstrate that cleavage by endo R takes place at the AAV-vector junction, we hybridized the products of the reaction to AAV and pBR322 probes (Fig. 3). The reaction conditions we chose were sufficient to cleave 90% of the substrate (form I pSM620) at least once. When the products of the reaction were examined by ethidium bromide staining without further digestion with a restriction enzyme, most of the product was linear plasmid DNA. In addition, approximately 3% of the products consisted of two fragments which were 4.7 and 4.4 kb long: the sizes expected for excised duplex AAV and the reciprocal linear pBR322 DNA (Fig 3A, lane R). Further digestion of the endo R products with either *Bst*EII or *Sph*I produced a set of fragments which demonstrated that the linear plasmid product of the endo R reaction was the result of cleavage at either one or the other AAV-pBR322 junction (Fig. 1 and 3). For example, when *Bst*EII was used, we saw six fragments. The largest (9.1 kb) comes from *Bst*EII cleavage of the circular (form II) plasmid DNA that survived the initial incubation with endo R. The remaining bands are 7.4, 6.1, 4.4, 3.0, and 1.7 kb. The 7.4- and 1.7-kb bands come from linear plasmid DNA that had been cleaved in the endo R reaction at the left AAV-vector junction (Fig. 1). The 6.1- and 3.0-kb bands come from initial endo R cleavage at the right end of AAV. Finally, the 4.4-kb pBR322 fragment has no *Bst*EII site and survives the *Bst*EII digestion. The origins of these fragments was confirmed by hybridization with AAV and pBR322 probes (Fig. 3B). The fragments that should consist exclusively of AAV sequences hybridized only with AAV probe, and, conversely, the

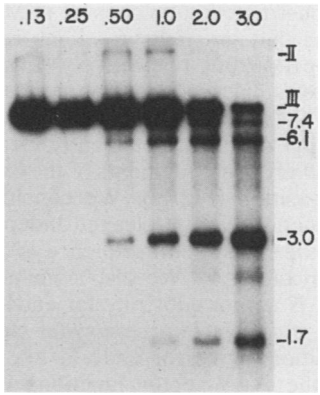

FIG. 2. Enzyme titration of fraction III. The numbers at the top refer to the number of units of endo R used in each reaction (see Materials and Methods). The sizes of the major cleavage fragments are indicated on the right. The standard endo R reaction mixture contained (in 25 μl) 0.5 μg of form I pSM620 plasmid DNA, 5 mM $MgCl_2$, 20 mM Tris hydrochloride (pH 7.5), 1 mM dithiothreitol, and the indicated amount of fraction III enzyme. After incubation for 1 h at 37°C, the reaction products were treated with phenol, precipitated with ethanol, and digested with *Bst*EII. The reaction mixture was then fractionated on a 1.4% agarose gel, transferred to a nitrocellulose filter, and hybridized to nick-translated ^{32}P-labeled AAV DNA.

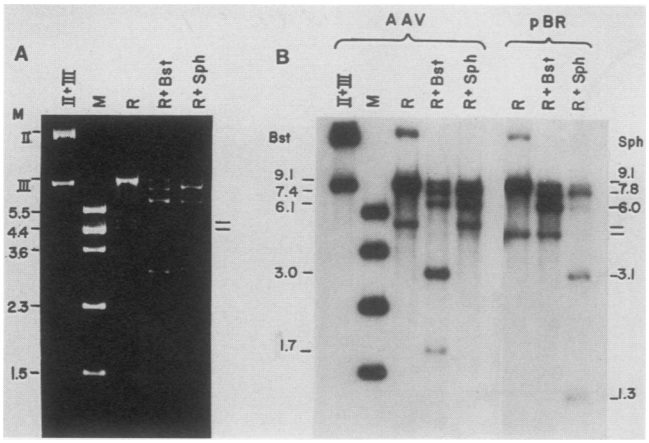

FIG. 3. Identification of AAV-specific and pBR-specific fragments in the endo R reaction. pSM620 substrate was incubated with fraction IV enzyme in a standard endo R reaction (lanes R). Where indicated, the reaction products were also digested with *Bst*EII or *Sph*I. The products were fractionated in duplicate on a 1.4% agarose gel, transferred to nitrocellulose filters, and hybridized to either AAV-specific or pBR-specific probe (panel B). The endo R-plus-*Bst*EII and endo R-plus-*Sph*I fragment sizes are indicated to the left and right, respectively, of panel B. Panel A illustrates the reaction products stained with ethidium bromide. Also indicated in panel A are the sizes and positions of the marker bands (lane M) and pSM620 relaxed circular (II) and linear (III) plasmid DNA (lane II + III). The two unidentified lines to the right of each panel indicate the position of linear AAV (upper) and linear pBR (lower) DNA.

fragments that were expected to have only pBR322 DNA hybridized only with pBR probe. We estimated that the sensitivity of the hybridization was sufficient to detect 50 bp of either AAV or vector sequences. Similar results were seen when the restriction enzyme *Sph*I was used instead of *Bst*EII (Fig. 1 and 3).

Either AAV-vector junction can be cleaved independently of the other. It was possible that cleavage by endo R was the result of an interaction between the two terminal repeats. To test this, we constructed subclones of pSM620 which contained only the left or right terminal repeat of AAV, pGM620C and pBM620D, respectively (Fig. 4). In addition to the 145-bp terminal sequence, both plasmids contained approximately 350 bp of flanking AAV DNA. Both pGM620C and pGM620D are efficiently cleaved by endo R (fraction III) and produce fragments of the expected size and sequence composition (Fig. 4). We concluded that either AAV-vector junction can be cleaved independently of the other and that the recognition sequence is contained in the terminal 500 bp of AAV. We also concluded that vector DNA, pBR322, is a poor substrate for endo R (Fig. 4).

Identification of sequences necessary for cleavage. To identify the recognition signal for endo R, we determined the sequences of the AAV-vector junctions in a number of mutant AAV plasmids and compared their relative frequencies of cleavage (Fig. 5; Table 1). We concluded that there were three sequences near the AAV-vector junctions that could serve as substrates. The first was a stretch of poly(G) residues that was present at many of the junctions and was a result of the fact that the plasmids had been constructed by G · C tailing. The second was the sequence CCaCT CCCTCTCT, which consists of nucleotides 3 to 15 of the AAV terminal repeat, and this was called the AAV recognition signal. Because the AAV terminal repeat is a palindrome, this sequence occurs twice within each wild-type

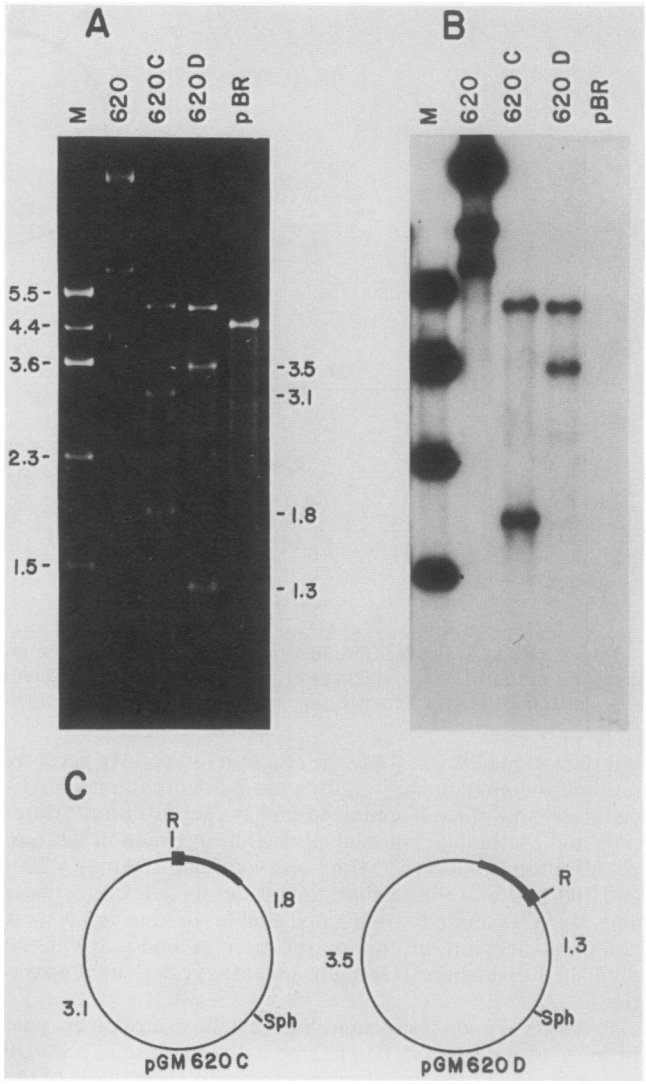

FIG. 4. Cleavage of substrates that contain only one copy of the AAV terminal repeat. Panel C shows maps of pGM620C and pGM620D. pGM620C contains the 513-bp left terminal *Pst*I fragment of the wild-type AAV plasmid, pSM620, including the left AAV terminal repeat and the 19-bp poly(g) tail. pGM620D includes the right terminal repeat of AAV and the 29-bp G · C tail in a 445-bp *Pst*I fragment subcloned from pSM620. Symbols: ▬, AAV DNA; ——, pBR DNA; ■, AAV terminal repeats. Also indicated are the positions of the endo R (R) and *Sph*I sites. In panels A and B, 0.5 μg of pGM620C (lanes 620C), pGM620D (lanes 620D), or pBR322 (lanes pBR) circular plasmid DNA was incubated with fraction III enzyme as described in the legend to Fig. 2 and digested with *Sph*I. The reaction products were fractionated in a 1.4% agarose gel, stained with ethidium bromide (panel A), and then transferred onto a nitrocellulose filter and hybridized to nick-translated AAV DNA probe (panel B). The positions of the marker bands (M) and the endo R-plus-*Sph*I product bands are indicated to the left and right, respectively, of panel A. Lanes 620 contain forms I, II, and III pSM620 plasmid DNA.

terminal repeat, nucleotides 3 to 15 and 109 to 121, and the two copies were called the outboard and inboard recognition sequences, respectively. The presence of the inboard copy of this sequence accounts for the minor cleavage band derived from the left end of AAV and seen at the 1.6-kb position in virtually all of the plasmids shown in Fig. 5. It is

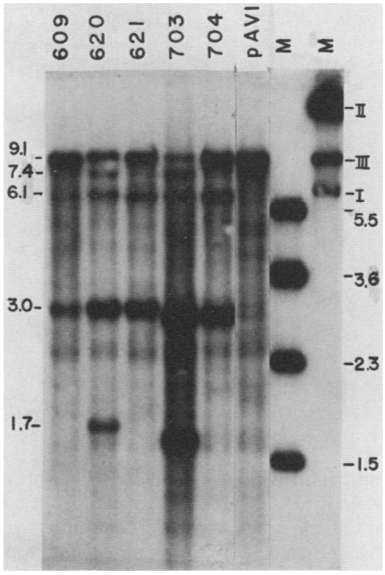

FIG. 5. Cleavage of AAV plasmids that contain deletions in their terminal repeats. The various mutant substrates were incubated with fraction III enzyme and *Bst*EII as described in the legend to Fig. 2 and probed with ^{32}P-labeled AAV DNA. The sequence at each AAV/pBR junction is listed in Table 1. The figure was overexposed to visualize minor cleavage bands. The 1.6-kb fragment of pSM703 has a slightly slower mobility owing to the presence of a 36-bp G · C tail (Table 1).

essentially the 1.7-kb endo R-*Bst*EII fragment minus the first 100 bp of AAV DNA, i.e., the distance between the outboard and inboard copies of the AAV recognition sequence. In three junctions [pSM621 (L), PSM704 (L), and pSM609 (L); Fig. 5 and Table 1] the AAV cleavage site is the only recognition sequence present at the junction, because the deletion in these plasmids has removed the poly(G) tail and placed the inboard copy of the sequence adjacent to vector DNA. Accordingly, the 1.6-kb band is the only fragment seen in these plasmids as the result of cleavage at the left end. In the pSM620 (L) junction, both copies of the AAV recognition sequence were present and both the 1.6- and 1.7-kb bands were produced. Presumably, the inboard site at the right end of AAV also is cleaved, but the separation in this region of the gel was not sufficient in most cases for the 2.9-kb fragment to be visible (Fig. 5).

The relative intensities of the 1.7- and 1.6-kb bands coming from pSM620 (L) reflect the presence of the poly(G)$_{19}$ adjacent to the outboard site. In general, the homopolymeric poly(G) tail sequence was approximately 20- tc 100-fold more likely to be cut than the heteropolymeric AAV recognition sequence. In contrast to pSM620, pAV1 contains no G · C tail and cleavage is directed exclusively by the AAV terminal recognition site. As a result, cleavage at the inboard and outboard sites occurs with approximately equal efficiency to produce two junction bands from each end of approximately equal intensity (Fig. 5, the fragment sizes are 3.0, 2.9, 1.7, and 1.6 kb). The fact that poly(G)$_n$ alone was sufficient to act as a recognition sequence was demonstrated by pSM703 (L) (Fig. 5; Table 1). This plasmid contains a deletion which has removed both the inboard and outboard AAV sites but retains a poly(G) stretch that is 36 bp long. The poly(G) stretch was sufficient to promote cleavage and produced a band of approximately 1.6 kb.

Minor cleavage sites. Cleavage by endo R appeared to require a sequence that was at least 10 bp long and consisted of a polypurine-polypyrimidine tract that was relatively rich in G · C base pairs. To confirm these requirements, we asked whether other minor cleavage sites in Fig. 5 could be predicted on the basis of this sequence. We noticed that a number of minor bands appeared in digests of all of the AAV variant plasmids and that these fragments must therefore have been the result of cleavage within AAV sequences. (If they had been the result of cleavage in the pBR322 DNA, they would have spanned the pBR-AAV junction and their size would have varied depending on the size of the terminal deletion.) Using a computer, we searched for similarities within the AAV sequence to either poly(G)$_{19}$ or the complementary sequence poly(C)$_{19}$. Table 2 lists the sequences and the predicted cleavage fragments that were generated by the computer search. There was good correspondence between the computer-predicted cleavage pattern and the fragments

TABLE 1. Recognition site for endo R[a]

Plasmid type	AAV-vector junction sequence	Relative frequency[b]
(g)$_n$ + CCaCTCCCTCTCT		
pSM620(L)	Vector-(g)$_{19}$ CCaCTCCCTCTCTgCgCg-AAV	100
pSM620(R)	Vector-(g)$_{29}$ CCaCTCCCTCTCTgCgCg-AAV	100
pSM621(R)	Vector-(g)$_{29}$ CCaCTCCCTCTCTgCgCg-AAV	100
pSM703(R)	Vector-(g)$_{38}$ CCaCTCCCTC| aCTaggg-AAV	150
pSM609(R)	Vector-(g)$_{10}$ | CTCCCTCTCTgCgCgCTC-AAV	50
CCaCTCCCTCTCT		
pSM621(L)	AAV-CCaCTCCCTCTCTgC | agC-Vector	1
pSM704(L)	AAV-CCaCTCCCTCTCTgC | agC-Vector	1
pSM609(L)	AAV-CCaCTCCCTCTCTgC | agC-Vector	1
(g)$_n$		
pSM703(L)	Vector-(g)36 | agTggCCaaCTCCaTCaC-AAV	150

[a] The first two bases of the AAV sequence in pSM620 (gg) are counted as part of the poly(g) tail. A vertical line (|) indicates the position of the deletion in the mutant. pSM704(R) and the pAV1 plasmid junctions were not sequenced. (R) and (L) stand for right and left junctions, respectively. pSM620 and pSM609 had been previously sequenced (43); the sequence of pSM609(L) was found to be incorrect and is changed here. The remaining plasmids were sequenced as part of this study.

[b] Indicates the approximate yield of the fragment resulting from cleavage at the indicated site (Fig. 5); 100% cleavage has been defined as the cleavage seen at pSM620 (R). No attempt was made to distinguish between junctions in each category.

TABLE 2. Minor cleavage sites[a]

Sequence	Position (nucleotide)	Fragment length (kb)	
		Predicted[b]	Observed[c]
CCCCTCTCCCCTC	4089	2.39 (10/13)	2.4
CCaCTCCCTCTCT	4678	3.00 (8/13)	3.0
CCaCTCCCTCTCT	4562	2.86 (8/13)	NC
CCaCTCCCTCTCT	3	1.70 (8/13)	1.7
CCaCTCCCTCTCT	121	1.59 (8/13)	1.6
CTCCaCCCCTCC	157	1.54 (9/12)	NC
CTaCagCaCCCCTT	3026	1.33 (7/14)	1.3
CCCTgCCCaCCT	2947	1.25 (8/12)	1.2
CagCagCCCCCTCT	2775	1.08 (8/14)	1.1
CCagaCTCCTCCTC	2661	0.96 (8/14)	ND
CCCgCCTCCggCgCC	753	0.95 (10/15)	ND
CCCCTCCTCCCaCC	1461	0.24 (11/14)	TS

[a] The predicted size was derived by assuming that cleavage occurred 3′ to the poly(G) sequence. All fragment sizes are the result of *Bst*EII digestion of endo R products.

[b] Ratios (in parentheses) refer to the numbers of G residues per lengths of recognition sequences.

[c] NC, Not clear; ND, not detected; TS, too small to be resolved on a 1.4% agarose gel.

seen in endo R digests. In fact, all of the observed fragments that were believed to be the result of cleavage in AAV DNA were predicted by the computer search. In particular, the best match we found was with a sequence of nucleotide 4089 which produces a 2.4-kb fragment.

Isolation of the cleavage site. It was still possible that endo R recognized an internal AAV sequence which was not part of the terminal repeats and then cleaved at the nearest stretch of homopurine-homopyrimidine that was available. To rule out this possibility, we constructed four additional plasmids. In the first plasmid, pGM1008, we subcloned the right junction of pSM620 and deleted all but the first 21 bp of AAV DNA. This plasmid was cleaved with approximately the same frequency as the parental junction (Table 3). In addition, we constructed two plasmids, pGMGC9 and pGMGC13, which contained poly(G)-poly(C) stretches of 9 and 13 bp, respectively, inserted at the *Pst*I site of pBR322 (Table 3). No AAV sequences were present in either plasmid. When these plasmids were incubated with endo R, we found that both the 9-bp and the 13-bp poly(G) plasmids were cleaved. The results obtained by using pGMGC9 and pGMGC13, as well as those obtained with the pSM609 (R) junction (Table 1), suggested that the minimum length of G residues sufficient for cleavage was 9 bp. Two naturally occurring stretches of poly$(G)_6$ in pBR322 were not detectably cleaved. Finally, we also constructed a plasmid,

TABLE 3. Subcloned cleavage sites[a]

Clone	Inserted sequence	Frequency
pGM1008	$(g)_{29}$ CCaCTCCCTCTCTgCgCg	100
pGMGC13	$(g)_{13}$	30
pGMGC9	$(g)_9$	20
pBR322[b]	$(g)_6$	0
pGMAAVter	$(g)_2$ CCaCTCCCTCTCTgCgCg	1

[a] Frequencies were defined as percent starting substrate cleaved in a standard assay with 1 U of endo R. The value for pGM1008 was arbitrarily set to 100%.

[b] Natural poly(G) stretches of 6 bp occur in pBR322 at nucleotides 2550 and 2797.

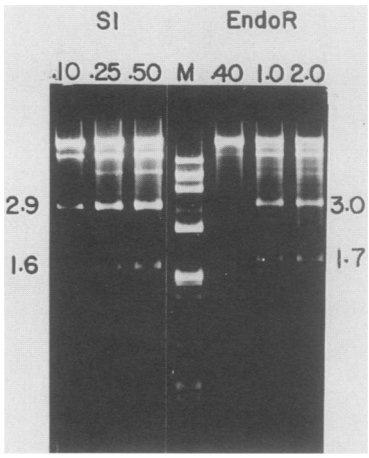

FIG. 6. S1 and endo R nuclease digestions of pSM620. pSM620 DNA (1.0 μg) was titrated with S1 nuclease or fraction IV endo R in a 50-μl reaction volume under standard conditions for each enzyme (see Materials and Methods). Numbers at the top indicate the number of units of each enzyme used.

pGMAAVter21, that contained only nucleotides 3 to 23 of the AAV terminal sequence (ggCCaCTCCCTCTCTgCgCgC) and contained the AAV terminal recognition sequence. This plasmid was also cleaved by endo R (Table 3).

Is endo R a single-stranded endonuclease? A number of single-stranded endonucleases will cleave polypurine-polypyrimidine stretches that are present in duplex DNA. The activity has been demonstrated for S1, mung bean, and *Neurospora* endonucleases, as well as for snake venom phosphodiesterase (9, 13, 39, 47). For short polypurine stretches, the reaction requires supercoiled DNA. To find whether endo R was a single-stranded DNA endonuclease we first compared the products of S1 and endo R digestions of pSM620 (Fig. 6). S1 produced a cleavage pattern similar to that seen with endo R. However, unlike endo R, S1 did not seem to discriminate between the inboard and outboard ends of the AAV terminal palindrome. It apparently cut equally well at both ends of the terminal repeat, producing a full-length pBR322 linear DNA molecule (4.4 kb) and the shorter 1.6- and 2.9-kb AAV fragments. (On these gels we would not have detected the remaining 100-bp fragment.) A similar experiment with mung bean endonuclease did not produce discrete bands (results not shown).

Having normalized the activities of endo R and S1 on duplex substrates, we then asked whether endo R was equally active on single-stranded *E. coli* DNA (Fig. 7). When equivalent amounts of S1 and endoR (as measured on duplex DNA) were incubated with ^{3}H-labeled single-stranded *E. coli* DNA, S1 converted 70% of the DNA to acid-soluble product within 30 min, whereas endo R produced no acid-soluble products after 2 h. The results were similar regardless of whether the reactions were carried out at pH 6.0 for both enzymes or whether each enzyme was assayed at its own pH optimum (pH 4.5 for S1 and pH 7.5 for endo R). On the basis of this assay, we estimated that endo R was approximately 50- to 100-fold less active on single-stranded DNA than S1 was. We also compared the ability of S1 and endo R to cut single-stranded circular M13 DNA. When assayed by gel mobility, endo R cut M13 DNA at a rate 20-fold lower than S1 did (results not shown). We do not know whether this activity on single-stranded circular DNA is an intrinsic property of endo R or the result of a residual contaminating endonuclease.

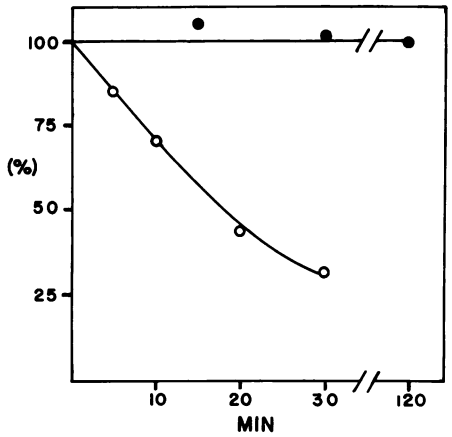

FIG. 7. S1 and endo R activity on single-stranded DNA. Equivalent amounts of double-stranded cleaving activity of S1 (1.25 U) or endo R (5.0 U) were incubated with 5.0 μg of single-stranded uniformly ^{3}H-labeled *E. coli* DNA in a 250-μl reaction volume at 37°C under standard reaction conditions for each enzyme (see Materials and Methods). At the indicated times, portions of each reaction mixture were acid precipitated and counted. The percent acid-precipitable counts remaining at each time is plotted on the vertical axis. Symbols: ●, endo R; ○, S1.

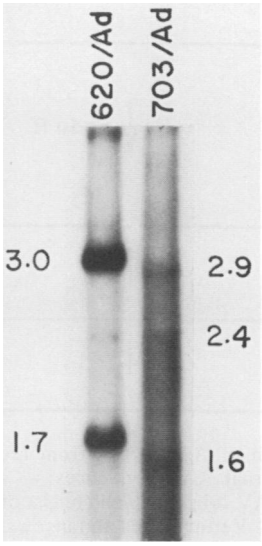

FIG. 9. Cleavage of AAV plasmids in vivo. pSM620 and pSM703 DNA (5.0 μg) were transfected into adenovirus-infected HeLa cells (see Material and Methods). At 21 h after transfection, low-molecular-weight DNA was isolated, digested with *Bst*EII, and electrophoresed and probed as described in the legend to Fig. 2.

Finally, we examined the ability of single- and double-stranded DNA to inhibit the cleavage of pSM620 by endo R (Fig. 8). Both single-stranded and duplex DNA stimulated endo R activity at low concentrations (competitor/substrate ratio, 1:4 to 1:1) and inhibited the activity at higher concentrations (2:1 to 4:1). At high concentrations, single-stranded DNA inhibited the activity approximately twofold more than double-stranded DNA did. When poly(G)$_n$-poly(C)$_n$ was added to the reaction, inhibition was seen at every ratio of inhibitor to substrate that was tried.

Does endo R cleave AAV plasmid DNA in vivo? Although we had demonstrated that the endo R activity was capable of excising AAV sequences in vitro, we could not be certain that it would cleave plasmid DNA in vivo. When AAV plasmids are transfected into tissue culture cells, the bulk of the input DNA is found in the form of nicked circular (form II) or linear (form III) plasmid species (18, 43, 45; J. Gottlieb and N. Muzyczka, unpublished results). This is true regardless of whether the input AAV DNA is capable of DNA replication. To find whether the linear plasmid DNA (form III) that is produced in vivo is the result of cleavage at a specific sequence, we digested that DNA with *Bst*EII and hybridized it with AAV probe (Fig. 9). The 1.7- and 3.0-kb bands were readily seen in cells transfected with the wild-type plasmid, pSM620 (Fig. 9), and in this case they were presumably derived from replicating AAV DNA. However, bands of the same size were seen at reduced levels in cells transfected with pSM620 in the absence of adenovirus or in the presence of adenovirus and hydroxyurea (results not shown). In addition, two minor bands (2.4 and 1.6 kb) were seen in cells transfected with pSM620; they apparently were not amplified by DNA replication. Both of these bands were expected on the basis of the cleavage pattern of endo R in vitro, and their origin has been discussed above. For pSM703, we expected junction fractions approximately 100 bp shorter than those seen with the wild-type plasmid as a result of deletions present within both ends of pSM703 (compare with Fig. 5). These bands were readily seen in cells transfected with pSM703 DNA (Fig. 9). Because pSM703 is a mutant (45), the observed 1.6- and 2.9-kb fragments must be derived from the input plasmid sequences. As expected, pSM703 also generated a 2.4-kb band. We concluded that a substantial amount of input plasmid DNA was cleaved at the AAV-vector junction in vivo and that AAV DNA replication is not necessary for cleavage to occur. Moreover, the pattern of cleavage was essentially the same in vivo and in vitro. The remaining input DNA was apparently cleaved randomly as judged by the background radioactivity.

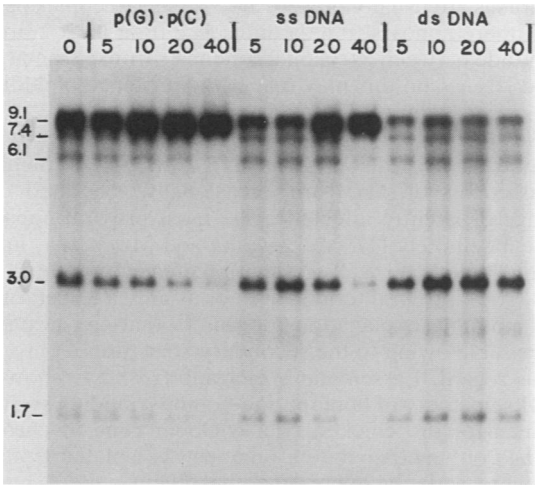

FIG. 8. Inhibition of endo R activity with single- and double-stranded DNA. pSM620 DNA (20 μg/ml) was incubated with fraction IV enzyme in a 50-μl standard reaction volume containing 5, 10, 20 or 40 μg of inhibitor DNA per ml. The inhibitors were poly(dG)-poly(dC) double-stranded DNA [p(G) · p(C)] heat-denatured *E. coli* DNA (ssDNA), or double-stranded *E. coli* DNA (dsDNA).

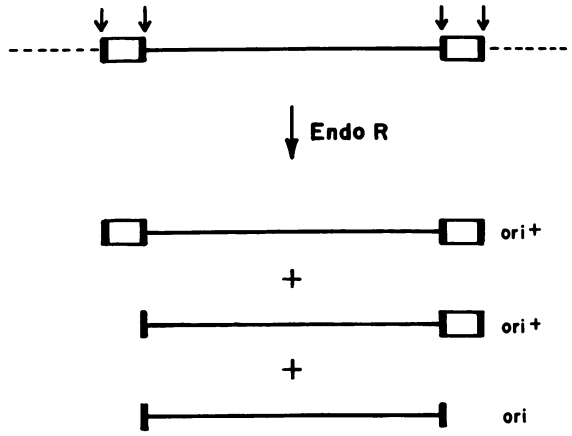

FIG. 10. Excision of AAV DNA from recombinant plasmids. Symbols: ▬, internal AAV sequences; - - - -, flanking plasmid sequences; ▭, AAV origins which consist of the terminal 125-bp palindromic repeats. Vertical lines and arrows indicate the inboard and outboard AAV recognition sequences and endo R cleavage sites.

DISCUSSION

Is endo R responsible for AAV excision in vivo? When AAV plasmids are transfected into human cells, most of the input plasmid DNA is converted to relaxed circular and linear plasmid DNA. This is true regardless of whether the plasmid contains an AAV genome capable of autonomous replication (18, 28, 43, 45) or whether the transfection is done in the presence of hydroxyurea (Gottlieb and Muzyczka, unpublished results). Digestion of the input DNA with restriction enzymes indicates that a large fraction of the input DNA has been cleaved at one or the other AAV junction with vector DNA. Additional fragments seen in vivo suggest that specific cleavage also occurs at the inboard copies of the AAV terminus (the sequence CCaCTCCCTCTCT) and at an internal sequence to produce fragments of 1.6 to 2.4 kb. This means that specific AAV sequences are substrates for a host-encoded endonuclease activity and that this activity does not require DNA replication. All of these in vivo observations are consistent with the properties of endo R in vitro. However, without the benefit of a cellular mutation in endo R, we cannot be certain that this activity is required for AAV rescue.

If endo R represents a major pathway for AAV rescue, then excision of AAV sequences by endo R must explain not only how wild-type AAV sequences are rescued, but also how mutants which contain terminal deletions are rescued. In view of the sequence specificity of endo R, we can offer the following model for rescue of wild-type and mutant sequences (Fig. 10). Normal AAV rescue occurs by endo R cleavage at the two outboard AAV recognition sites to directly produce an AAV replicative intermediate. This can occur when the wild-type plasmid pSM620 or pAV1 is transfected into mammalian cells. In some molecules cleavage can occur at an outboard site in one end and only at an inboard site at the other end (pSM621 and pSM609). These molecules can generate an AAV replicative intermediate by subsequent correction of the missing *ori* sequence (45). However, molecules that have been cleaved at both inboard endo R sites are *ori* and are incapable of DNA replication. Such molecules would have to recombine with uncleaved input plasmid molecules to recover a functional origin sequence. A prediction based on this model is that the deletion of the terminal 15 bp of AAV from both ends (i.e., deletion of both outboard recognition sites) should significantly reduce the infectivity of AAV plasmids. This is, in fact, consistent with the behavior of a mutant plasmid (p*sub*201+) recently reported by Samulski et al. (44), which is missing the first 13 bp from both ends.

The frequency of endo R excision of AAV plasmids is also consistent with what is seen in vivo. On the basis of the frequency of cleavage in vitro, we would expect that only 0.01 to 1% of the input pSM620 molecules would be cut at both junctions to produce *ori*$^+$ AAV duplex DNA. In a typical transfection experiment (0.1 to 10 μg of plasmid DNA per 10-cm dish), we estimate that an excision frequency of 0.01% would produce 0.3 to 30 AAV duplex DNA molecules in each transfected cell (see Materials and Methods for calculation). This is approximately the limit of detection in most transfection experiments. However, if this frequency is correct, there should be a linear relationship between the yield of replicative-form DNA produced in transfected cells and the amount of input plasmid DNA used in the transfection. In agreement with this, Samulski et al. (43) have shown that the yield of AAV DNA is linear with the amount of input DNA in the range between 0.1 and 10 μg of plasmid DNA per 10-cm dish.

In using the recombinant plasmids as substrates for in vitro excision, we have assumed that these plasmids were models for AAV proviruses integrated into chromatin. Typically, a cell line carrying AAV proviruses contains two to five tandem copies of the AAV genome (11, 26; McLaughlin and Muzyczka, in preparation). The junction of two tandem AAV copies would contain the palindromic sequence aga gagggagTggCC/ggCCaCTCCCTCTCT, i.e., a stretch of polypurine residues followed by a polypyrimidine stretch. This is a derivative of the sequence at the junction of pBR322 and AAV in pSM620 (g_n/CCaCTCCCTCTCT) and should be a substrate for endo R. Qualitatively, however, there must be at least two major differences between rescue from chromatin and rescue from recombinant plasmids. First, although approximately 10^3 to 10^4 molecules of AAV plasmid DNA are required per cell for maximum production of AAV virions in a transfection experiment, endogenous AAV proviruses are usually present at less than 10 copies per cell. Thus, there appears to be a difference of at least 1 order of magnitude between excision frequencies from chromatin and naked DNA, and this may reflect the presence of additional components required for excision from chromatin.

A second issue is the apparent stability of AAV proviruses in the absence of helper virus (11). Although extrachromosomal copies of AAV are occasionally generated during passage of latently infected cells, most proviral copies are stable indefinitely (11; McLaughlin and Muzyczka, in preparation) unless the cell is superinfected with helper virus. Thus, AAV proviral termini are not usually a target for endo R. A possible explanation for this is that the termini are inaccessible owing to the secondary structure of chromatin. In this regard, Larsen and Weintraub (25) have shown that the S1 sensitivity of homopurine-homopyrimidine sequences upstream of the chicken beta-A-globin gene in chromatin depends on whether the chromatin was isolated from cells that were actively expressing the globin gene. Thus, the initiation of AAV rescue by adenovirus superinfection may be due to the activation of AAV gene expression by adenovirus gene products (27, 40).

Properties of endo R. The best substrate for endo R in vitro is poly(g)$_n$-poly(C)$_n$. Substitution of g residues with A is tolerated, but results in significantly lower cleavage frequen-

cies, e.g., for the AAV terminal recognition sequence. The alternating copolymer poly(ga)$_{38}$-poly(CT)$_{38}$ cloned in a pBR322 derivative (13) is cleaved at a frequency about 10-fold lower than the poly(g)$_{13}$ stretch in pGMGC13, and poly(A)-poly(T) cleavage cannot be detected in any of the substrates we have tried (Gottlieb and Muzyczka, in preparation). Furthermore, the minimum length of the recognition stretch is 9 bp. No cleavage was detected at stretches of poly(g) or poly(ga) shorter than 9 bp in any of the plasmid substrates used in this study. In stretches longer than 10 bp, only two pyrimidine-for-purine substitutions were allowed (Table 2).

The activity profile of endo R is strikingly similar to the activity of a number of single-stranded DNA endonucleases. For S1, it seems clear that the enzyme is recognizing an altered secondary structure in duplex DNA that exists at stretches of polypurine-polypyrimidine DNA (9, 13, 39). Local melting of the DNA strands or strand shifting to produce single-stranded loops does not appear to be involved (9). The extract nature of this non-B, non-Z DNA structure is not clear, but is is possible that, like S1, endo R recognizes this structure as a substrate for cleavage.

We could not detect significant amounts of any other type of endo- or exonucleolytic activity associated with endo R. In particular, we could not detect specificity for single-stranded DNA by acid-solubilization or competition assays. The small amount of single-stranded DNA cutting that we detected on M13 single-stranded circles was probably due to residual amounts of a contaminating endonuclease which were present in the phosphocellulose fraction (fraction III). Further purification of endo R should resolve the question of whether the enzyme has any intrinsic activity on substrates other then duplex poly(g)$_n$.

We note that researchers in several other laboratories have isolated endonucleases from eucaryotic cells that show at least some specificity for G residues (12, 24, 32, 42). In some respects endo R is different from all of these activities. Despite these differences, it is possible that endo R is related to some and perhaps all of these previously described enzymes. We note that none of these enzymes has been purified to homogeneity and that the properties of endo R have changed significantly during the course of our own purification. By analogy with the RecBC endonuclease (33, 41, 52, 53), it is possible that all of these endonucleases share a common core activity whose specificity is modified by inhibitory or accessory proteins or the conditions of the reaction.

What does endo R do for the cell? The isolation from HeLa cells of an enzyme whose sole activity appears to be cleavage at homopurine-homopyrimidine sequences raises the question of the role of these sequences in cellular DNA. Endo R is one of three major endonucleolytic activities that we have detected in HeLa nuclei (Gottlieb and Muzyczka, unpublished). However, because of the specificity of endo R, it is unlikely that it plays a degradative role in the cell. Furthermore, the results of Larsen and Weintraub (25) suggest that most poly(g) or poly(ga) sequences in chromatin would be protected from cleavage unless they are undergoing transcription or replication.

The distribution of homopurine-homopyrimidine sequences appears to be predominantly in noncoding sequences (2, 20, 23, 38), but their distribution does not suggest any obvious role. An intriguing aspect of these sequences is that in many cases they have been found in regions of genetic instability, either as part of satellite DNA (14) or as part of variable-number tandem repeat units (20, 23, 38). One commonly proposed mechanism for amplifying or reducing the number of repeats in a tandem array is unequal homologous recombination, and this has led to the suggestion that variable-number tandem repeat sequences may contain hot spots for recombination (23). Although there is no direct evidence for this, we find it striking that many of the variable-number tandem repeats have homopurine-homopyrimidine stretches similar to those found in AAV. It is possible, then, that endo R recognizes these variable-number tandem repeat sequences and is involved in one of the cellular pathways for recombination. If, in fact, AAV termini contain a recognition sequence for cellular recombination, this might explain how AAV proviruses are both integrated and rescued.

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LITERATURE CITED

1. **Atchison, R. W., B. C. Casto, and W. M. Hammond.** 1965. Adenovirus-associated defective virus particles. Science **149:** 754–756.
2. **Bell, G. I., M. J. Selby, and W. J. Rutter.** 1982. The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences. Nature (London) **295:** 31–35.
3. **Berns, K. I., N. Muzyczka, and W. W. Hauswirth.** 1985. Replication of parvoviruses, p. 415–432. *In* B. Fields (ed.), Virology. Raven Press, New York.
4. **Berns, K. I., T. C. Pinkerton, G. F. Thomas, and M. D. Hoggan.** 1975. Detection of adeno-associated virus (AAV)-specific nucleotide sequences in DNA isolated from latently infected Detroit 6 cells. Virology **68:**556–560.
5. **Boissy, R., and C. R. Astell.** 1985. *Escherichia coli recBC sbcB recF* hosts permit the deletion-resistant propagation of plasmid clones containing the 5′-terminal palindrome of minute virus of mice. Gene **35:**179–185.
6. **Bolivar, F., R. L. Rodriguez, D. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1979. Construction and characterization of new cloning vectors. II. A multipurpose cloning system. Gene **2:**95–113.
7. **Boyer, H. W.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–479.
8. **Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Rose.** 1981. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J. Virol. **40:**241–247.
9. **Cantor, C. R., and A. Efstradiatis.** 1984. Possible structures of homopurine-homopyrimidine S1-hypersensitive sites. Nucleic Acids Res. **12:**8059–8072.
10. **Challberg, M. D., and T. J. Kelly.** 1979. Adenovirus DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **76:**655–659.
11. **Cheung, A. K., M. D. Hoggan, W. W. Hauswirth, and K. I. Berns.** 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. J. Virol. **33:**739–748.
12. **Desiderio, S., and D. Baltimore.** 1984. Double-stranded cleavage by cell extracts near recombinational signal sequences of immunoglobulin genes. Nature (London) **308:**860–862.
13. **Evans, T., and A. Efstradiatis.** 1986. Sequence-dependent S1

nuclease hypersensitivity of a heteronomous duplex. J. Biol. Chem. **261**:14771–14780.
14. **Fowler, R. F., and D. M. Skinner.** 1986. Eucaryotic DNA diverges at a long and complex pyrimidine-purine tract that can adopt altered conformations. J. Biol. Chem. **261**:8994–9001.
15. **Handa, H., K. Shiroki, and H. Shimojo.** 1977. Establishment and characterization of KB cell lines latently infected with adeno-associated virus type 1. Virology **82**:84–92.
16. **Hauswirth, W. W., and K. I. Berns.** 1977. Origin and termination of adeno-associated virus DNA replication. Virology **78**: 488–499.
17. **Hauswirth, W. W., M. J. van de Walle, P. J. Laipis, and P. D. Olivo.** 1984. Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue. Cell **37**:1001–1007.
18. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. **51**:329–339.
19. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
20. **Hoffman-Liebermann, B., D. Hoffman-Liebermann, A. Troutt, L. H. Kedes, and S. N. Cohen.** 1986. Human homologs of TU transposon sequences: polypurine/polypyrimidine sequence elements that can alter DNA conformation in vitro and in vivo. Mol. Cell. Biol. **6**:3632–3642.
21. **Hoggan, M. D., G. F. Thomas, F. B. Thomas, and F. B. Johnson.** 1972. Continuous carriage of adenovirus associated virus genome in cell culture in the absence of helper adeno-virus, p. 243–249. *In* Proceedings of the Fourth Lepetite Colloquium. North-Holland Publishing Company, Amsterdam.
22. **Holloman, W. K., T. C. Rowe, and J. R. Rusche.** 1981. Studies on nuclease alpha from *Ustilago maydis*. J. Biol. Chem. **256**:5087–5094.
23. **Jeffreys, A. J., V. Wilson, and S. L. Thein.** 1985. Hypervariable "minisatellite" regions in human DNA. Nature (London) **314**: 67–73.
24. **Kataoka, T., S. Kondo, M. Nishi, M. Kodaira, and T. Honjo.** 1984. Isolation and characterization of endonuclease J: a sequence-specific endonuclease cleaving immunoglobulin genes. Nucleic Acids Res. **12**:5995–6010.
25. **Larsen, E., and H. Weintraub.** 1982. An altered DNA conformation detected by S1 nuclease occurs at specific regions in active chick globin chromatin. Cell **29**:609–622.
26. **Laughlin, C. A., C. B. Cardellichio, and H. C. Coon.** 1986. Latent infection of KB cells with adeno-associated virus type 2. J. Virol. **60**:515–524.
27. **Laughlin, C. A., N. Jones, and B. J. Carter.** 1982. Effect of deletions in adenovirus region 1 genes upon replication of adeno-associated virus. J. Virol. **41**:868–876.
28. **Laughlin, C. A., J. D. Tratschin, H. Coon, and B. J. Carter.** 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene **23**:65–73.
29. **Lefebvre, R. B., S. Riva, and K. I. Berns.** 1984. Conformation takes precedence over sequence in adeno-associated virus DNA replication. Mol. Cell. Biol. **4**:1416–1419.
30. **Lusby, E., K. H. Fife, and K. I. Berns.** 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. J. Virol. **34**:402–409.
31. **Maxam, A. M., and W. Gilbert.** 1977. A new method of sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
32. **McKenna, W. G., J. J. Maio, and F. L. Brown.** 1981. Purification and properties of a mammalian endonuclease showing site-specific cleavage of DNA. J. Biol. Chem. **256**:6435–6443.
33. **Muskavitch, K. M. T., and S. Linn.** 1981. RecBC-like enzymes: exonuclease V deoxyribonucleases, p. 233. *In* P. D. Boyer (ed.), The enzymes, vol. 14. Academic Press, Inc., New York.
34. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of SV40 DNA with DEAE-dextran. J. Natl. Cancer Inst. **41**:351–357.
34a. **McLaughlin, S. K., P. Collis, P. L. Hermonat, and N. Muzyczka.** 1988. Adeno-associated virus general transduction vectors: analysis of proviral structures. J. Virol. **62**:1963–1973.
35. **McPherson, R. A., L. J. Rosenthal, and J. A. Rose.** 1985. Human cytomegalovirus completely helps adeno-associated virus replication. Virology **147**:217–222.
36. **Mocarski, E. S., and B. Roizman.** 1981. Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. Proc. Natl. Acad. Sci. USA **78**:7047–7051.
37. **Muzyczka, N.** 1980. Construction of an SV40-derived cloning vector. Gene **11**:63–77.
38. **Nakamura, Y., M. Leppert, P. O'Connell, R. Wolfe, T. Holm, M. Culver, C. Martin, E. Fujimoto, M. Hoff, E. Kumlin, and R. White.** 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science **235**:1616–1622.
39. **Pulleyblank, D. E., D. B. Haniford, and A. R. Morgan.** 1985. A structural basis for S1 nuclease sensitivity of double-stranded DNA. Cell **42**:271–280.
40. **Richardson, W. D., and H. Westphal.** 1985. Adenovirus early gene regulation and the adeno-associated virus helper effect. Curr. Top. Microbiol. Immunol. **109**:147–165.
41. **Rosamond, J., K. M. Telander, and S. Linn.** 1979. Modulation of the action of the recBC enzyme of *Escherichia coli* K-12 by Ca^{2+}. J. Biol. Chem. **254**:8646–8652.
42. **Ruiz-Carillo, A., and J. Renaud.** 1987. Endonuclease G: $(dG)_n$-$(dC)_n$-specific DNAse from higher eukaryotes. EMBO J. **6**:401–407.
43. **Samulski, R. J., K. I. Berns, M. Tan, and N. Muzyczka.** 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. Proc. Natl. Acad. Sci. USA **79**:2077–2081.
44. **Samulski, R. J., L. S. Chang, and T. Shenk.** 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. J. Virol. **61**:3096–3101.
45. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33**:135–143.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
47. **Schon, E., T. Evans, J. Welsh, and A. Efstradiatis.** 1983. Conformation of promoter DNA: fine mapping of S1-hypersensitive sites. Cell **35**:837–848.
48. **Senapathy, P., J. D. Tratschin, and B. J. Carter.** 1984. Replication of adeno-associated virus DNA. Complementation of naturally occurring rep^- mutants by a wild-type genome or an ori mutant and correction of terminal palindrome deletions. J. Mol. Biol. **179**:1–20.
49. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
50. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45**:555–564.
51. **Straus, S. E., E. D. Sebring, and J. A. Rose.** 1976. Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated DNA synthesis. Proc. Natl. Acad. Sci. USA **73**:742–746.
52. **Taylor, A. F., D. W. Schultz, A. S. Ponticelli, and G. R. Smith.** 1985. RecBC enzyme nicking at chi sites during DNA unwinding: location and orientation dependence of cutting. Cell **41**:153–163.
53. **Taylor, A. F., and G. R. Smith.** 1980. Unwinding and rewinding of DNA by the recBC enzyme. Cell **22**:447–457.