

Adeno-Associated Virus Autointerference

BARRIE J. CARTER,¹ CATHERINE A. LAUGHLIN, LUIS M. DE LA MAZA,
AND MAUREEN MYERS

*Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism, and Digestive Diseases,
Building 4, Room 316, National Institutes of Health, Bethesda, Maryland 20014*

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We have analyzed an autointerference phenomenon exhibited by adeno-associated virus type 2 (AAV) when grown in KB cells coinfecting with adenovirus type 2 as the helper. Infectious AAV particles that banded at 1.41 g/cm³ in CsCl were purified by three cycles of centrifuging in CsCl equilibrium gradients. When cells were infected with an increasing multiplicity of these AAV particles there was a corresponding decrease in production of infectious progeny AAV. There was also an AAV multiplicity-dependent inhibition of production of infectious adenovirus and inhibition of Ad DNA replication. The viral DNA in the Hirt supernatant fraction extracted from cells infected with different multiplicities of AAV was analyzed in neutral sucrose gradients. At low multiplicities of infection with AAV, the main AAV DNA species synthesized was the mature 14.5 S (standard) viral genome. In higher multiplicity infections with AAV increasing amounts of aberrant 10 S AAV DNA molecules accumulated and the proportion of 14.5 S AAV DNA decreased. Restriction endonuclease cleavage showed that the 10 S DNA was enriched for the left- or right-hand terminal regions of the AAV genome. These molecules may be analogous to the previously characterized aberrant DNA molecules found in light-density AAV particles. Thus, the AAV autointerference is correlated with production of the aberrant deleted AAV genomes.

INTRODUCTION

Most parvoviruses produce several classes of particles as assayed by buoyant density equilibrium sedimentation in CsCl gradient [reviewed in Rose (1974) and Siegl (1976)]. The defective human parvovirus, adeno-associated virus (AAV) grown in human (Hoggan, 1971) or monkey cells (B. Carter, unpublished results), using a human adenovirus as the helper, produces two classes of infectious particles: a predominant component which bands in CsCl at a density of 1.41 g/cm³ and a minor component at a density of 1.45 g/cm³. In addition, a spectrum of lighter, noninfectious AAV particles is produced of which the predominant classes band at densities of 1.35 and 1.32 g/cm³. These virus classes will be referred to as AAV(1.41), AAV(1.45),

AAV(1.35), and AAV(1.32) particles, respectively (de la Maza and Carter, 1978).

We have begun a systematic study of the physical and biological properties of these various AAV particles to assess their significance in the viral replication cycle. Elsewhere we have reported an analysis of the physical structure of DNA in each class of AAV particle (de la Maza and Carter, 1978). Briefly, the densities of the AAV particles which are lighter than 1.41 g/cm³ are proportional to the size of the encapsidated DNA molecule. The genomes of these lighter density particles contain deletions but retain the left or right-hand terminal regions of the DNA and many have a "snap-back" or hairpin duplex conformation at neutral pH. Also, within the population of infectious virus particles which band at 1.41 or 1.45 g/cm³ there are some particles which contain deleted, snap-back genomes.

¹ To whom reprint requests should be addressed.

In this report we describe experiments to document and analyze an autointerference phenomenon exhibited by AAV(1.41) particles. Specifically we show that the autointerference phenomenon is correlated with the level of aberrant DNA replication which results in production of the deleted AAV genomes. The aberrant, deleted DNA molecules that were previously observed in encapsidated virions may be analogous to those observed in the population of intracellular AAV DNA molecules. Preliminary accounts of this work have appeared elsewhere (American Society for Microbiology Abstracts 1978, p. 229; Fourth International Virology Congress, 1978, Abstracts p. 534).

MATERIALS AND METHODS

Virus and cells. Stocks of adeno-associated virus type 2 (AAV-2H) and adenovirus type 2 were grown in KB-3 cells in suspension culture at 37°. AAV(1.41) particles were purified from cell lysates using the trypsin-deoxycholate procedure (Berns and Rose, 1970) followed by three cycles of banding in CsCl equilibrium bouyant density gradients as described elsewhere (L. de la Maza and B. Carter, in preparation). All AAV preparations used for experiments were heated at 60° for 15 min to inactivate any contaminating adenovirus.

In all the experiments to analyze viral DNA synthesis, KB cells were grown in monolayer cultures in Eagle's medium containing 5% fetal calf serum. Cell lines are routinely checked by standard procedures for absence of mycoplasma contamination. Cells were grown to 50–70% confluency then infected with Ad2, and an appropriate multiplicity of AAV. Virus dilutions were made in phosphate-buffered saline (PBS) pH 7.2, containing 2% dialyzed fetal calf serum. Adsorption of virus was performed in a 2.0-ml volume at room temperature for 1 hr. The inoculum was then removed, 10 ml of Eagle's medium containing 2% dialyzed fetal calf serum was added, and the infected cultures were incubated at 37°. For experiments using cells on microscope

slides adsorption of virus was performed as described below.

Isotopic labeling and extraction of intracellular viral DNA. Infected cell cultures (75 cm² T-flasks) were labeled with [methyl-³H]thymidine (specific activity, 40–60 Ci/mM, New England Nuclear Corp.) at a concentration of 25 to 50 μ Ci/ml for 4 hr at 16 to 20 hr after infection. The medium was then removed and the cells lysed by a modification of the method of Hirt (1967). The cell sheet was washed twice with ice-cold PBS then 2.5 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)] was added and the culture incubated for 30 min at 37°. Pronase (Calbiochem, B-grade, nuclease free, suspended at 10 mg/ml in 10 mM Tris-HCl, pH 8.0, and predigested for 2 hr at 37° was added to a final concentration of 500 μ g/ml and incubation continued at 37° for 2 hr. The lysed culture was then carefully poured into a glass centrifuge tube and 5 M NaCl was added to a final concentration of 1.1 M. The mixture was gently inverted several times, allowed to stand at 4° overnight, and then centrifuged at 10,000 rpm for 20 min in the HB-4 rotor of the Sorvall RC-5 centrifuge. The supernatant was dialysed for 72 hr against three changes of 4 liter of either 0.15 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA or 1 $\times$ SSC, 1 mM EDTA. Under these conditions the AAV DNA usually reannealed an to ensure this the dialyzed DNA was incubated at 66° for 1 hr prior to velocity sedimentation.

Velocity sedimentation of viral DNA. Intracellular DNA preparations were sedimented in 12.0-ml 5 to 20% sucrose gradients containing 1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Gradients were centrifuged in nitrocellulose tubes in the Beckman SW41 rotor for 14–17 hr at 28,000 rpm and 20° in either a Beckman ultracentrifuge or a Sorvall OTD 65 ultracentrifuge. Gradients were collected by bottom puncture using a constant-volume device. Whole gradient fractions or aliquots were counted in toluene/Triton scintillation fluid to determine radioactivity.

Restriction endonuclease cleavage and

electrophoresis of DNA. Fractions containing ^3H -labeled DNA were pooled from sucrose gradients, dialyzed, and concentrated by vacuum dialysis to remove sucrose then extracted with phenol and precipitated with ethanol. The precipitated DNA was resuspended in buffer containing 6 mM MgCl_2 , 6 mM 2-mercaptoethanol, 6 mM Tris-HCl (pH 8.0) and cleaved by incubation for 2 hr at 37° with several units of endonuclease *Hae*III or *Hha*I (Biolabs, Beverly, Mass.). For *Hha* cleavage 50 mM NaCl was included in the reaction. The endonuclease digests were adjusted to 1% SDS and electrophoresed in a 3 to 10% acrylamide gradient gel. The gel was then fluorographed according to Bonner and Laskey (1974) to detect ^3H -labeled DNA bands.

Assay of AAV infectivity. Infectious units of AAV were measured using a fluorescent focus assay. KB cells were grown in a monolayer on microscope slides containing eight chambers (Lab-Tek Products, Naperville, Ill.). Each chamber contained 5×10^5 cells. The cells were infected with the helper adenovirus at a multiplicity of infection (m.o.i.) of 5 plaque-forming units (PFU)/cell and an appropriate dilution of the AAV preparation. Virus dilutions were made in Eagle's medium without serum and absorption was allowed to proceed for 1 hr at room temperature in a volume of 0.1 or 0.2 ml/chamber. The inoculum was then removed, fresh medium (1.0 ml) containing 2% fetal calf serum was added, and the cultures were incubated at 37° in a CO_2 incubator. Cells were fixed at 30 hr after infection by removing the medium and immersing in 95% methanol, 5% PBS at room temperature for 10 min, then air dried and stored at -20° until stained as below.

The number of cell nuclei containing AAV antigen was then determined using an indirect immunofluorescent assay. The fixed cells were rinsed with PBS then incubated with anti-AAV-2 rabbit IgG for 45 min at 37° . The cells were then washed thoroughly with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, Ind.) for 45

min at 37° . The cells were then washed again with PBS and covered with one drop of PBS-glycerol (2:3) and a coverslip. The proportion of cells exhibiting nuclear fluorescence was determined using a Zeiss microscope equipped with uv-epifluorescent optics.

When adenovirus is used as the helper for AAV the maximum proportion of infected cell nuclei that can be rendered fluorescent for AAV is usually 50–65%. Therefore, according to the Poisson equation, we take the amount of the virus dilution that gives 63% of this maximal number of infected cell fluorescent nuclei as being equivalent to a multiplicity of one infectious unit of AAV per cell: i.e., 0.1 ml of the AAV dilution that gives 63% of this maximal nuclei fluorescence contains 5×10^5 infectious units.

The anti-AAV-2 rabbit IgG used for the assays was an IgG fraction purified from a hyperimmune rabbit serum raised against intact AAV-2 particles and will be described more fully elsewhere. For controls the IgG fraction of normal rabbit serum was used.

Assay of adenovirus infectivity. Infectivity of adenovirus was measured by a tissue culture infectious dose assay measuring nuclei fluorescent for adenovirus capsid antigens performed in an analogous way to that described above for AAV. The first antiserum used was a rabbit anti-adenovirus-2 obtained from Microbiological Associates (Bethesda, Md.). Adenovirus infectivity was also measured using a plaque-formation assay on monolayers of KB or HeLa cells at 37° . Cell monolayers were prepared in 35-mm wells and a 0.5-ml inoculum of virus was adsorbed for 75 to 90 min at 37° . The inoculum was removed and the cultures were immediately overlaid with 3.5 ml of Eagle's medium containing 15% fetal bovine serum and 0.9% Noble's agar. An additional 2.5 ml of overlay medium was added after 7 days incubation at 37° . At 12–14 days postinfection 2 ml of the above overlay medium containing 0.01% neutral red was added. Plaques were scored 4 days after staining.

Both assays gave similar values for adenovirus infectivity.

RESULTS

*Autointerference of AAV (1.41) Particles:
Biological Assays*

KB cells were infected at various multiplicities of AAV(1.41) and the proportion of nuclei which were fluorescent for AAV antigens was determined. In addition, the yield of infectious AAV progeny was assayed. The results are summarized in Fig. 1 and clearly show that there is a decrease in the yield of infectious AAV progeny as the multiplicity of infection is increased above 10. The increase of AAV yield until a multiplicity of 10 is reached is due to infection of a greater proportion of the cells with AAV (Fig. 1). The maximum number of cells infected with AAV as determined by assay of fluorescent nuclei is usually 50–65% but can be increased to 75–80% if the cells are infected with AAV several hours prior to infection with adenovirus. The failure to infect 100% of the cells with AAV is not understood but may be related to AAV autointerference, the failure of adenovirus to induce sufficient helper factor(s) in every cell or competition by adenovirus. The AAV autointerference is more readily demonstrated by assaying the

yield of infectious virus produced rather than the proportion of nuclei fluorescent for AAV antigen. This indicates that autointerference for AAV occurs in the bulk of the infected cells rather than in a particular subset.

Analysis of Viral DNA in Infected Cells

Viral DNA synthesis in infected cells was analyzed by incorporation of [³H]thymidine from 16 to 20 hr after infection followed by selective extraction of viral DNA by the method of Hirt (1967). The relatively long 4-hr labeling period was chosen so that progeny DNA molecules would predominate over replicating form (RF) molecules. Also, 16 to 20 hr after infection is the period of maximum rates of progeny DNA and virion accumulation for both AAV and adenovirus. (Rose, 1974)

The sedimentation analysis of viral DNA from infected cells is shown in Fig. 2. The amount of ³H label incorporated into DNA that was recoverable in the Hirt supernatant of mock-infected cells was only 1 to 2% of the amount in virus-infected cells (see legend to Fig. 2). In adenovirus-infected cells the predominant DNA species

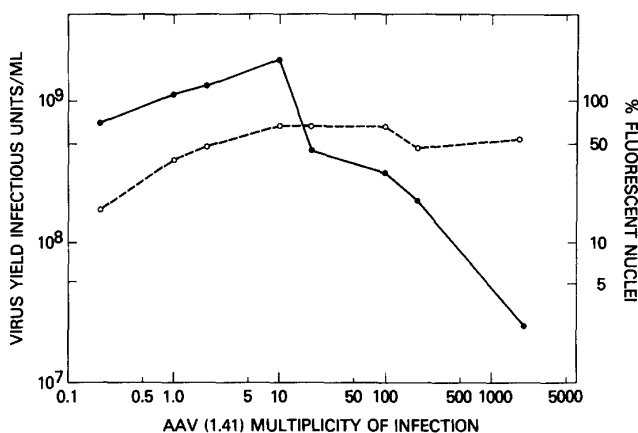


FIG. 1. KB cells in monolayer wells on microscope slides were infected with adenovirus type 2 at a m.o.i. of 10 PFU/cell and with multiplicities of AAV (1.41) virus as indicated. For each dilution of AAV (1.41) four wells of cells were infected. Two wells were fixed at 30 hr postinfection and stained for AAV fluorescent nuclei (open circle, broken line). The other two wells were harvested at 40 hr and the yield of infectious virus was determined (solid circle, solid line).

was the 31 S native Ad2 DNA duplex as well as some faster sedimenting Ad2 replicating intermediates. The amount of ^3H label from Ad2-infected cells that sedimented more slowly than 20 S was no greater than that from the mock-infected cells. In cells infected jointly with adenovirus and AAV the amount of ^3H -labeled adenovirus 31 S DNA was decreased (by at least 70%) and there were several new DNA species which included major peaks at 14.5 and 9 to 10 S and a shoulder at 15 to 20 S. These new species, which have previously been shown to be AAV DNA (Strauss *et al.*, 1976; Hauswirth and Berns, 1978), were characterized further by velocity sedimentation and restriction endonuclease cleavage.

Structure of AAV DNA Species in Hirt Supernatant Fraction

Two cultures of KB cells were infected with AAV(1.41) at multiplicities of 0.2 or 10.0, respectively, and Ad2 at 5 PFU/cell. ^3H -labeled viral DNA was selectively extracted from each culture and sedimented in a neutral sucrose gradient. Infection with a low multiplicity of AAV (Fig. 3a) yielded a large amount of 31 S Ad DNA and a peak of 14.5 S AAV DNA. The high-multiplicity infection (Fig. 3e) showed a similar amount of 14.5 S AAV DNA, a large amount of a 9–10 S species and a 90% inhibition of 31 S Ad DNA synthesis. Various fractions from these two neutral sucrose gradients were pooled as indicated, dialyzed, concentrated, and resedimented in both neutral and alkaline sucrose gradients.

The 14.5 S DNA (pool 2) sedimented mainly as mature unit length AAV DNA molecules in both neutral (Fig. 3c) and alkaline (Fig. 3g) gradients. The alkaline gradient does show that 10% of pool 2 consisted of single strands sedimenting at the rate of dimer length AAV RF molecules (1.27-fold faster than unit length) that are cross-linked and snap back at neutral pH (Strauss *et al.*, 1976). This presumably represents cross-contamination of pool 2 by pool 1. Pool 3, which is the 14.5 S DNA

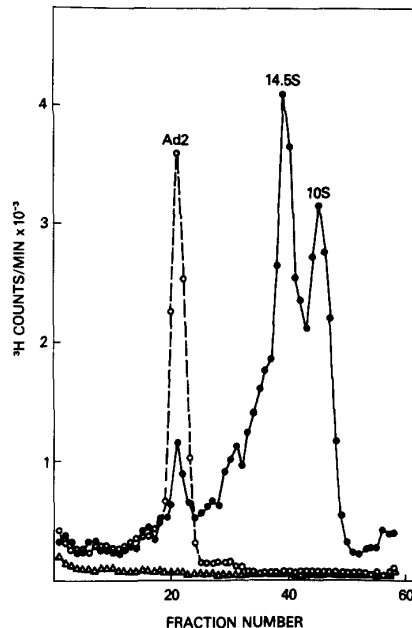
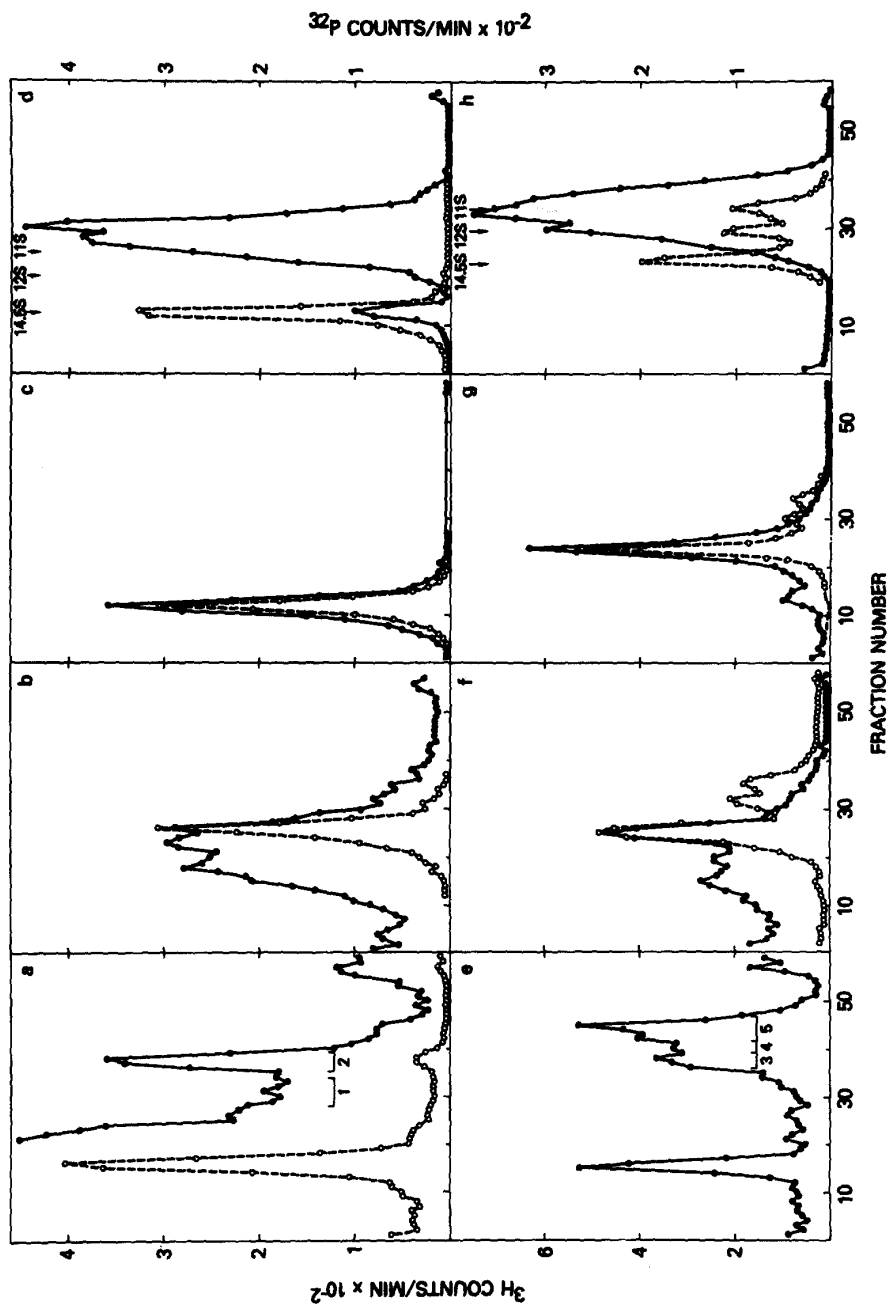


FIG. 2. Sedimentation in neutral sucrose of DNA from the Hirt supernatant fraction of infected cells. Cells were infected with virus and intracellular DNA was labeled with [^3H]thymidine and extracted as described under Materials and Methods. Aliquots of DNA from mock-infected cells (Δ), cells infected with Ad2 at an m.o.i. of 5.0 PFU/cell ($\circ$), or cells infected with Ad2 (m.o.i., 5) + AAV-2 (1.41) m.o.i. = 10 ($\bullet$) were sedimented in parallel gradients. The position of markers were determined by sedimenting ^{32}P -labeled AAV DNA in each gradient (profile not shown). The sedimentation profiles from parallel sucrose gradients have been superimposed. The total amounts of ^3H radioactivity (cpm) incorporated into 50 μl of Hirt supernatant extract were 2587 (mock-infected cells), 105,087 (Ad2 infected), and 158,965 (Ad2 + AAV infected). Sedimentation in this and succeeding figures is from right to left.

from the high-multiplicity infection showed exactly analogous behavior to pool 2 in both neutral and alkaline gradients (data not shown). Pool 1 is expected to contain AAV replicating molecules (Strauss *et al.*, 1976), and as shown in Figs. 3b and f, most of these molecules sedimented faster than unit length in both neutral and alkaline sucrose. The peak of unit length molecules from pool 1 that is observed in alkaline sucrose



(Fig. 3f) is about 30% of the total ^3H label and is derived in part from cross-contamination from pool 2 and from the presence in pool 1 of duplex oligomeric species formed by "cohesive-end" annealing of unit length AAV DNA (Carter and Khoury, 1975; Koczot *et al.*, 1973; Gerry *et al.*, 1973).

Pool 5 contained the 9–10 S DNA which in neutral sucrose (Fig. 3d) sedimented as duplex molecules of 10 to 30% unit length but in alkaline sucrose (Fig. 3h) contained strands of 20 to 60% unit length. This DNA may therefore consist, in part, of snap-back molecules analogous to those from light-density particles which we have previously characterized (de la Maza and Carter, 1978). Further experiments, which are currently in progress, are required to clearly demonstrate this.

Pools of DNA similar to those as described in Fig. 3 were analyzed by restriction endonuclease cleavage as illustrated by the example shown in Fig. 4 using *Hae*III and *Hha*I. Pool 3 (14.5 S) DNA yielded the *Hae*III and *Hha* fragments in equimolar yield as expected for mature AAV DNA duplexes. The pool 5 material (9–10 S DNA) clearly yielded a nonequimolar distribution of fragments (Fig. 4). The *Hae*III fragments D and E, located near the right and left molecular end, respectively, are clearly enriched for and the next most abundant fragments are the immediately adjacent internal fragments, F, at the right end and N at the left end. That the *Hae*III component at positions C and D in pool 5 is mainly D is confirmed by the absence, or relative deficiency, of the fragments G and J in the *Hha* digest of pool 5. The internal

*Hae*III fragments A, B, G, H, I, J, K, L, and M are absent or greatly deficient in pool 5.

The *Hha* digest of pool 5 (Fig. 4) complements the *Hae*III cleavage. *Hha* cleavage yielded fragment H from the left molecular end, a component with a size between that of *Hha* A and B from the right end (corresponding to the *Hae*III fragments D and F), and a fragment with the mobility of *Hha* K, L, or M which are not well resolved in this gel. This latter fragment probably is mainly *Hha* K from the inverted repetition at each end since the regions containing *Hha* L (i.e., *Hae*III J and A) and *Hha* M (i.e., *Hae*III C) are absent from, or greatly deficient in, the *Hae*III cleavage of pool 5 (Fig. 4). The longer exposure of the *Hha* cleavage shows additional heterogeneous material migrating at the rate of *Hha* B, C, and D. This presumably indicates the heterogeneity in size of these molecules as also indicated by the gradient of decreasing fragment abundance from the termini to the middle of the genome.

The material in pool 4 which represents DNA from the trough between 14.5 S and 10 S shows an intermediate cleavage pattern (Fig. 4) with an increased abundance of more internal fragments. This probably represents the larger size of the molecules (i.e., extending further towards the center of the genome) and some contamination from 14.5 S DNA. However, even pool 4 DNA shows a relative enrichment for terminal regions of the genome.

In summary, the cleavage data clearly show that the aberrant 10 S AAV DNA molecules are enriched for the terminal regions at the left- or right-hand ends and

FIG. 3. Sedimentation analysis of AAV Hirt supernatant DNA. ^3H -labeled DNA extracted from the Hirt supernatant of AAV(Ad)-infected cells was sedimented in neutral sucrose gradients. (a) Neutral sucrose gradient, cells infected with AAV (1.41) at m.o.i. = 0.2. The broken line shows the same data as the solid line but plotted on a 10-fold-decreased scale. (e) Neutral sucrose gradient, cells infected with AAV (1.41) at m.o.i. = 10.0. Regions of gradients shown in (a) or (e) were pooled as indicated and analyzed by sedimentation in neutral or alkaline sucrose gradients. Panels (b), (c), and (d) show neutral sucrose gradient profiles of pools 1, 2, and 5, respectively (solid line), and ^{32}P -labeled linear AAV2 DNA (14.5 S) as marker (broken line). The positions of *Eco*RI-A (12 S) and RI-B (11 S) markers were determined from a parallel gradient. Panels (f), (g), and (h) show alkaline sucrose gradient profiles of pools 1, 2, and 5, respectively (solid line), and ^{32}P -labeled AAV-linear DNA and ^{32}P -labeled AAV *Eco*RI-A and *Eco*RI-B fragments as markers (broken line). The three markers and their sedimentation constants 14.5, 12, and 11 S, respectively, are indicated by the arrows in (d) and (h). Sedimentation is from right to left.

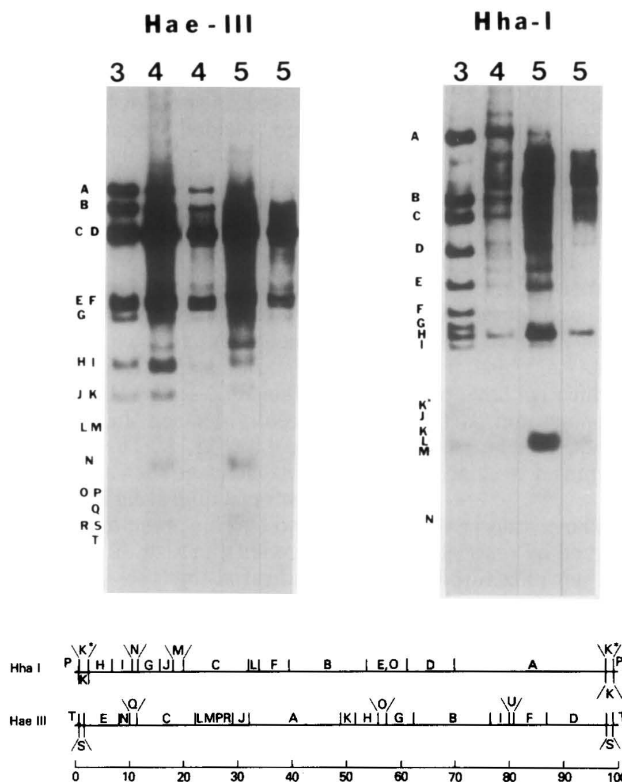


FIG. 4. Restriction endonuclease cleavage of AAV DNA in the Hirt supernatant of infected cells. DNA pools analogous to those of fractions 3, 4, and 5 of Fig. 3e were taken from a similar experiment and the DNA dialyzed, extracted with phenol, reprecipitated with ethanol, resuspended in appropriate buffer, and incubated with the restriction endonuclease *Hae*III or *Hha*I (de la Maza and Carter, 1977). The cleavage of fractions corresponding to 3, 4, and 5 of Fig. 3e is shown in tracts 3, 4, and 5, respectively, of the fluorogram. Pool 3 shows, as indicated, the fragments characteristic of AAV duplexes cleaved with *Hae*III or *Hha* (de la Maza and Carter, 1977) as indicated. The two tracts labeled 5 for *Hae*III and *Hha* and the two tracts labeled 4 for the *Hae*III show fluorographic exposures of the same gel for different lengths of time. All the reactions were performed at the same time and analyzed on the same gel slab. *Hha* O and P have migrated off the gel. The maps are drawn to scale in terms of percent of genome length.

particularly for those regions to the left of 10 to 20 or to the right of 80 to 90 on the map (Fig. 4). In this respect, these aberrant molecules resemble those previously characterized from light-density AAV particles (de la Maza and Carter, 1978). The cleavage patterns of the 10 S DNA (pool 5) shown in Fig. 4 do not appear to show the enrichment for the most out-board region of the terminal repetition (e.g., *Hae*III T) that might be expected based on the enrichment for the neighboring fragments. A similar

phenomenon was observed for AAV DNA from light-density AAV particles (de la Maza and Carter, 1978) and may mean that replication of those molecules terminates prematurely resulting in heterogeneity in the molecular termini. That these aberrant deleted molecules can be encapsidated also suggests that if encapsidation requires recognition of a specific AAV DNA sequence, then this sequence may be at or near the inverted repetition. It should also be noted that the cleavage pattern shows

that most of the 14.5 and 10 S DNA is AAV specific with little contamination with adenovirus DNA.

Autointerference Exhibited by AAV(1.41) Particles: DNA Replication

We have shown above (Fig. 1) that infections with AAV(1.41) particles exhibited autointerference. The data shown in Figs. 3a and e imply that infection at increasing multiplicity of AAV(1.41) results in a relatively increased amount of the shorter 10 S species of AAV DNA. To quantitate this observation further we performed a more extensive series of experiments. Cells were infected with varying multiplicities of AAV (1.41) particles and the viral DNA was labeled with [^3H]thymidine, selectively extracted, and analyzed in neutral sucrose gradients. From each gradient, the total amounts of radioactivity in the AAV 14.5 S peak and in the 10 S regions were calculated. The relative amount of 14.5 S DNA was calculated by dividing the 14.5 S radioactivity by the sum of 14.5 S + 10 S radioactivity. The product was expressed as a percentage and is plotted in Fig. 5B as a function of the multiplicity of AAV(1.41). Also shown in Fig. 5A is the sum of the 14.5 S + 10 S radioactivity which is a measure of total AAV DNA synthesis for completed molecules. The AAV replicating molecules sedimenting faster than 14.5 S were excluded from this calculation.

As shown by the analysis in Fig. 5B, increase in the multiplicity of infection of AAV(1.41) resulted in the synthesis of a decreasing proportion of standard 14.5 S DNA. Since the total amount of 14.5 S plus 10 S AAV DNA was fairly constant over the multiplicity range shown (Fig. 5A), the decreasing proportion of 14.5 S DNA represents an actual decrease in amount. The autointerference observed previously was therefore also exhibited at the level of DNA synthesis. Furthermore, this interference at the level of DNA synthesis was observed at a multiplicity as low as 1 infectious unit/cell.

The results shown in Fig. 5 were calculated from data obtained in neutral sucrose

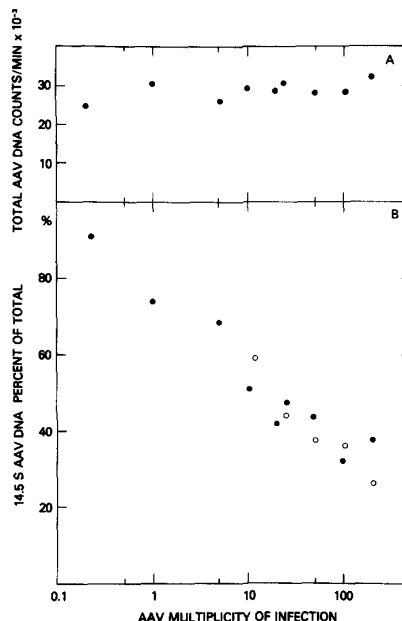


FIG. 5. Autointerference in AAV DNA replication. Flasks, 75 cm², of KB monolayer cells were coinfectd with Ad2 at a multiplicity of 2–3 PFU/cell and AAV (1.41) or AAV(1.45) at the multiplicities indicated. Aliquots of ^3H -labeled Hirt supernatant DNA isolated from each culture (labeled 16–20 hr postinfection) were sedimented in neutral sucrose gradients. The radioactivity in each of the regions containing mature standard 14.5 S AAV DNA (equivalent to pool 2 or 3 in Fig. 3) or the short DI DNA (equivalent to regions 4 and 5 in Fig. 3e). The sum of the radioactivity in these two regions was determined. The proportion of standard DNA was then determined as a percentage of the total radioactivity in both standard and DI-DNA and was plotted as function of AAV multiplicity. (A) Total AAV radioactivity for AAV(1.41) infection. (B) Percentage of total AAV DNA radioactivity present as standard 14.5 S DNA (●) AAV (1.41), (○) AAV (1.45).

gradients. This might result in a slight underestimate of the amount of standard 14.5 S DNA since some of these duplexes may form oligomeric species and sediment at the rate of replicating molecules (Carter and Khoury, 1975; see Fig. 3 above). Alternatively, some cross-linked dimer replicating strands may snap back and sediment at the rate of 14.5 S monomer duplexes (Straus *et al.*, 1976). However, a

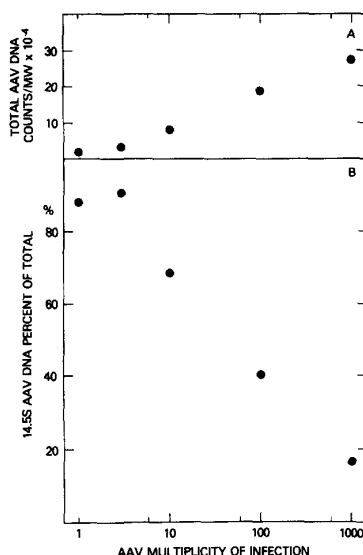


FIG. 6. Autointerference in AAV DNA replication. The experiment was performed as described in the legend to Fig. 5 except that only AAV (1.41) particles were used and the adenovirus multiplicity was 10 to 15 PFU/cell. (A) Total AAV DNA radioactivity for AAV(1.41) infection (i.e., sum of 14.5 S plus 10 S DNA cmp) (B) Percentage of total AAV DNA radioactivity present as 14.5 S DNA.

similar analysis (not shown) of data obtained from alkaline sucrose gradients yielded equivalent results. The AAV(1.45) particles were not studied so extensively but the experiment shown in Fig. 5 suggests that they also exhibit autointerference.

In the experiment shown in Fig. 5, the adenovirus helper was used at a multiplicity of 2–3 PFU/cell. Figure 6 shows a similar experiment in which the adenovirus multiplicity was 10–15 PFU/cell. Again the proportion of 14.5 S DNA decreased relative to that of 10 S DNA as the AAV multiplicity was increased (Fig. 6B). However, in contrast to the experiment of Fig. 5, the total AAV DNA synthesis (Fig. 6A) increased by about 10-fold over the multiplicity range (1 to 1000) of AAV(1.41) particles. Thus, although the proportion of 14.5 S DNA decreased with AAV multiplicity the actual amount of 14.5 S DNA

increased about eightfold and the amount of 10 S DNA increased about 240-fold.

The large increase in overall AAV DNA synthesis in Fig. 6 relative to that in Fig. 5 may reflect the lesser inhibition of adenovirus DNA synthesis by AAV at higher multiplicities of Ad (see Fig. 7). This in turn might reflect a maximal amount of viral DNA which can be made in any infected cell. Also, comparison of Figs. 5 and 6 suggests that the autointerference phenomenon may occur less readily at a higher adenovirus multiplicity, i.e., to obtain a given proportion of 14.5 S DNA a higher multiplicity of AAV may be required at higher multiplicities of adenovirus. The production of 10 S DNA molecules may depend on the level of an adenovirus helper function. Although the experiments in Figs. 5 and 6 were not performed on parallel cell cultures they do suggest that adenovirus multiplicity may have a quantitative rather than qualitative effect on AAV autointerference. A more precise quantitation of this is currently in progress.

Inhibition of Adenovirus Replication by AAV(1.41)

As shown in Table 1, the presence of increasing multiplicities of AAV(1.41) par-

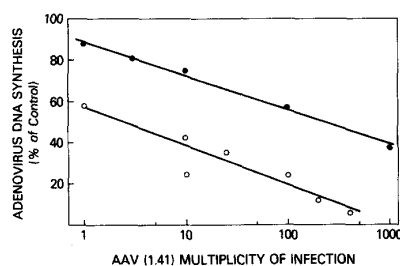


FIG. 7. KB cell monolayers were infected with AAV (1.4) at the multiplicity as indicated and with adenovirus at a multiplicity of 2.5 (○) or 10.0 (●) PFU/cell. The amount of adenovirus DNA synthesis was determined from the radioactivity in 31 S Ad2 DNA when the Hirt supernatant DNA (labeled with [³H]thymidine from 16 to 20 hr after infection) was sedimented in neutral sucrose gradients. The amount of 31 S adenovirus DNA is presented as the percentage of that in control cells infected only with adenovirus.

TABLE 1
INHIBITION OF ADENOVIRUS PRODUCTION BY AAV

Virus multiplicity ^a			Adenovirus infectivity yield ^c	
Adenovirus PFU/cell	AAV(1.41) TCID/cell	Time of harvest ^b (hr postinfection)	PFU/ml	Percentage of control
10	—	4	5×10^5	0.4
10	—	24	1.2×10^6	100.0
10	1	24	3.2×10^7	26.0
10	1000	24	$<10^6$	<1.0

^a KB cell monolayers in 35-mm wells were infected with adenovirus and AAV(1.41) at the multiplicities indicated as described under Materials and Methods.

^b The cells were harvested in 2.5 ml of culture medium by three cycles of freezing and thawing and clarified by low-speed centrifugation.

^c Clarified cell lysates were assayed for adenovirus plaque formation as described under Materials and Methods.

ticles resulted in proportional inhibition of adenovirus production. This inhibition was also analyzed at the level of DNA synthesis. As seen in Fig. 7, increasing multiplicities of AAV(1.41) clearly cause a progressive inhibition of synthesis of adenovirus 31 S progeny DNA as determined by neutral sucrose sedimentation of Hirt supernatant DNA from infected cells. The inhibition of adenovirus DNA synthesis appears to be rather less marked than the inhibition of virus production. This may mean either that AAV competes with, or inhibits, adenovirus both at the level of viral DNA synthesis and at some later stages such as transcription, translation, or viral assembly or that there is a cascade effect resulting from depression of adenovirus DNA synthesis.

DISCUSSION

The main conclusion to be made from the experiments described here is that a multiplicity-dependent autointerference can be observed in purified preparations of AAV(1.41) or AAV(1.45) particles. This autointerference is observed as a decreased yield of infectious progeny virus. The AAV stocks used for those experiments were stored in CsCl and heated to inactivate contaminating adenovirus (see Methods). If appropriate dilutions of CsCl or heat-inactivated adenovirus or both were added

to infected cells there was no inhibition of AAV (data not shown). Thus the autointerference does appear to be due to AAV particles. The interference appears to operate at least at the level of viral DNA synthesis and is correlated with a multiplicity-dependent decrease in synthesis of progeny 14.5 S AAV DNA and a corresponding increase in the amount of aberrant, deleted 10 S AAV DNA. It is an implicit assumption in this work that incorporation of [³H]thymidine reflects the actual amount of intracellular DNA synthesis rather than multiplicity-dependent changes in utilization of [³H]thymidine. A somewhat similar phenomenon to that described here for AAV has been reported by Muller *et al.* (1978). They showed that when the autonomous parvovirus *LuIII* is grown in HeLa cells there was a multiplicity-dependent production of light-density, D particles containing DNA which was smaller than the normal *LuIII* genome.

The shorter 10 S AAV DNA molecules obtained in the Hirt supernatant of infected cells were characterized by sedimentation analysis and gel electrophoresis following restriction endonuclease cleavage. These experiments showed that the 10 S species is a population of deleted molecules, enriched at the left- or right-hand molecular ends of the AAV genome. These molecules may be analogous to those which we

previously characterized from purified virus particles (de la Maza and Carter, 1978). Similar aberrant molecules of AAV DNA have been observed in Hirt supernatants of infected cells by Hauswirth and Berns, (1978) and probably account for the "dead-end" molecules reported by Strauss *et al.* (1976). The collective evidence argues for generation of these molecules by a replication mechanism rather than by a subsequent recombination or degradation during encapsidation. It is not yet established if all the aberrant, slowly sedimenting AAV DNA molecules present in the Hirt supernatant can in fact be encapsidated or if encapsidation occurs at the same efficiency as for standard 14.5 S DNA molecules. If encapsidation of AAV DNA requires recognition of a specific sequence then this sequence may be near the molecular termini.

The mechanism of the AAV autointerference phenomenon is not established by the experiments reported here but two general models can be distinguished. In the first model the interference might be due to defective-interfering (DI) particles (Huang and Baltimore 1970, 1977) present in the virus preparation. A second explanation is that the interference might be caused, not by AAV DI particles, but by some intrinsic phenomena related to increased AAV multiplicity such as viral overload of cellular replication machinery or a specific host-cell response to increased levels of some viral products. These two models are not mutually exclusive.

According to the first model it should be possible to remove any DI particles by successive low-multiplicity passages or to accumulate them by successive high-multiplicity passages. Also, if all aberrant or inhibitory particles were removed by low-multiplicity passage this would allow a direct test of the second model. Such experiments are currently in progress and suggest that the first model, involving AAV DI particles is a more likely possibility. One example of autointerference according to the second type of model would be interferon induction but this is unlikely because AAV neither induces interferon (Casto *et al.*, 1967; Parks *et al.*, 1968) nor

is sensitive to its action (H. Handa, R. Friedman, and B. Carter, unpublished results).

If DI particles are involved in the auto-interference phenomenon then the most likely candidates for such particles are those containing the aberrant deleted genomes which we have previously characterized (de la Maza and Carter, 1978). In numerous isotopically labeled preparations of AAV-2 particles made from the same passage level stock at the same multiplicity as the preparation used in the experiments described here we routinely find that approximately 5–10% of the virus particles obtained in the AAV(1.41) pool contained deleted, snap-back genomes. If these particles are DI particles, their proportion would be consistent with the fact that the autointerference was seen only at levels above a multiplicity of 0.5 to 1.0. Also, the relative proportion of these aberrant particles would imply that (a) they do not interfere at the level of adsorption and (b) they interfere very efficiently.

In order to assess directly the role of AAV particles containing snap-back, and/or deleted genomes as DI particles it is necessary to purify them free of infectious AAV particles. For the AAV particles with the largest aberrant DNA molecules this is difficult to do since these particles band in CsCl close to the density of infectious AAV(1.41) as mentioned above. However, in the analogous AAV particles banding at lighter density (particularly 1.35 g/cm³) in CsCl that also have deleted, genomes can be purified essentially free from any infectious standard AAV. Elsewhere we will report experiments which suggest that these lighter AAV particles do behave as DI particles (C. Laughlin, M. Myers, and B. Carter, in preparation).

In various sets of experiments with the same AAV stock there was some variation in the actual proportions of 14.5 S DNA and shorter 10 S DNA synthesized at any particular multiplicity. This variation may reflect variations in the precise metabolic state of the cells at the time of infection. Preliminary experiments in fact show that the proportion of short 10 S AAV DNA

synthesized depends both on the metabolic state of the cell and on the particular host-cell type. This suggests that the degree of aberrant replication and auto-interference exhibited by AAV may be influenced by the level of some host-cell factor. We have not yet systematically analyzed the influence of the adenovirus multiplicity on the AAV autointerference phenomenon. However, experiments reported in Figs. 5 and 6 suggest that the level of synthesis of aberrant AAV DNA molecules may be influenced by the adenovirus multiplicity and extent of inhibition of adenovirus by AAV.

One interesting feature of the experiments reported here is the size of the aberrant DNA molecules which are produced from infections with AAV(1.41). The predominant aberrant molecules which accumulate (Fig. 3e) appear to be about 9–10 S in size. Physical characterization and restriction cleavage of these DNA molecules (Figs. 3 and 4) suggest that they are analogous to those in AAV (1.35) particles (de la Maza and Carter, 1978). The aberrant molecules present in the AAV(1.41) particle preparations are somewhat larger than 10 S with a size range of 11–13 S in neutral sucrose (de la Maza and Carter, 1978). Thus, during replication either additional new (smaller) species can be produced or the smaller aberrant molecules, thought present in AAV(1.41) populations in very low amount, (if at all), may replicate preferentially over larger aberrant molecules.

An additional point demonstrated by these experiments is the inhibition of adenovirus by AAV. As shown by the yields of virus, this inhibition is multiplicity dependent. Furthermore, as shown in Hirt supernatants, this inhibition occurs, at least in part, at the level of DNA replication. Others have also shown previously that yields of adenovirus are decreased in the presence of AAV (Casto *et al.*, 1967; Parks *et al.*, 1968). It is interesting to note in this context that AAV(1.41) particles behave with respect to adenovirus as if they were adenovirus DI particles. Thus, AAV requires adenovirus as a helper

and when present in adenovirus stocks, initially at titers which are orders of magnitude lower than that of the adenovirus, it will with repeated high-multiplicity passage outgrow the adenovirus and decrease the adenovirus titer. This suggests the possibility that perhaps AAV arose as a DI particle of an ancestral adenovirus.

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