

Assembly of Adeno-Associated Virus

MAUREEN W. MYERS AND BARRIE J. CARTER¹

*Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism, and Digestive Diseases,
National Institutes of Health, Bethesda, Maryland 20205*

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The assembly of the defective parvovirus, adeno-associated virus (AAV), was studied by labeling DNA and protein precursors with [³H]thymidine or [³⁵S]methionine, respectively. Virus particles were extracted from cell lysates and characterized by banding to equilibrium in CsCl gradients and by velocity sedimentation in neutral sucrose. Radioactive pulse-chase labeling experiments showed that newly synthesized AAV proteins are rapidly assembled into empty capsids which in turn serve as precursors to stable, mature (full) particles that contain either a standard or deleted (defective-interfering) virus genome. While association of the newly synthesized progeny DNA strands with empty capsids appears to be rapid, maturation to form a stable full particle apparently is a much slower process requiring several hours. A new component, having the properties of an AAV genome associated with the empty capsid in a DNase-sensitive conformation (in contrast to the complete DNase resistance of DNA in mature particles), also exhibited pulse-chase kinetics consistent with a role as an assembly intermediate. L-Canavanine inhibited production of empty capsids and thus inhibited assembly of full particles. Pretreatment of infected cultures with hydroxyurea inhibited appearance of full particles and caused accumulation of empty capsids consistent with their precursor role. Addition of hydroxyurea immediately after a pulse label showed that maturation of AAV particles appears to occur in the absence of concomitant DNA replication. These experiments are consistent with a model for AAV assembly in which progeny DNA strands are packaged into preformed capsids.

INTRODUCTION

Parvoviruses consist of a single-stranded DNA genome encapsidated in a protein shell. Unlike other eucaryotic DNA-containing viruses, the parvoviruses apparently do not possess an internal core structure of histones or basic proteins. Therefore, the mechanism by which parvovirus DNA is encapsidated is of interest and may perhaps have some similarity with assembly of certain bacteriophages.

Adeno-associated virus (AAV) is a defective parvovirus which requires a helper adenovirus in order to replicate. The complementary plus and minus DNA strands of AAV are encapsidated in separate particles (Rose *et al.*, 1969). The precise nature of the helper function supplied by adenovirus is unknown.

When AAV particles from lysates of human KB cells are banded in CsCl gradients, the predominant particles obtained are referred to as AAV(1.45), AAV(1.41), AAV(1.35), and AAV(1.32) where the buoyant density of the particle in CsCl is written in parentheses (Carter *et al.*, 1979). Only the most dense particles, AAV(1.45) and AAV(1.41), contain standard full-length genomes and are infectious. Lighter density particles contain deleted genomes and are defective-interfering (DI) particles (de la Maza and Carter, 1977; Laughlin *et al.*, 1979). The lightest density class, AAV(1.32), includes particles containing only 1-5% of the viral genome which cannot be separated from completely empty AAV particles on the basis of their buoyant density in CsCl.

We have analyzed several parameters of AAV assembly in the context of two questions. Is the viral genome condensed into a preformed capsid or is the capsid as-

¹ To whom reprint requests should be addressed.

sembled from condensation of protein subunits around the genome? Does encapsidation of the viral genome require concomitant genome replication? Our studies suggest assembly of AAV by condensation of DNA into preformed capsids and show that at least part of this process apparently can occur in the absence of concomitant DNA synthesis.

METHODS

Virus and Cells. Stocks of adeno-associated virus type 2 (AAV2), wild-type adenovirus type 2 (H2wt), and wild-type adenovirus type 5 (H5wt) were grown in KB-3 cells in suspension culture at 37° and harvested at 40 to 48 hr after infection. AAV preparations were heated at 60° for 15 min to inactivate contaminating adenovirus. Adenovirus H2ts215 (obtained from W. Doerfler) is a late mutant of adenovirus type 2 which synthesizes DNA and at least some late proteins but does not produce mature particles at the restrictive temperature of 40° (M. W. Myers, unpublished). H2ts215 is an equally efficient helper for AAV replication at both permissive (32°) and nonpermissive (40°) temperatures (M. W. Myers, C. A. Laughlin, and B. J. Carter, unpublished). Stocks of the mutant were grown in KB-3 cells at 32°. Adenovirus and AAV infectivity were determined as described before (Carter *et al.*, 1979).

Monolayer cultures of KB cells in Eagle's medium containing 10% fetal calf serum were grown to 60–80% confluency and infected with adenovirus (5 PFU/cell) and AAV (1 infectious unit/cell). After 75–90 min of adsorption, the inoculum was removed, the cultures were rinsed once with Eagle's medium and then incubated at the appropriate temperature (37° with Adwt or 40° with Adts215) with fresh medium containing 2% fetal calf serum.

Isotopic labeling and extraction of viral particles and intracellular DNA. When [³⁵S]methionine (10–20 μ Ci/ml; specific activity 900–1100 Ci/mmol) was used in pulse-chase labeling experiments, unlabeled methionine in the medium was reduced to 0.3 μ g/ml. The chase medium contained unlabeled methionine (75 μ g/ml). After a [³H]-

thymidine pulse at a final concentration of 20–40 μ Ci/ml (specific activity 40–60 Ci/mmol) the chase medium contained unlabeled thymidine (25 μ g/ml).

Viral DNA was extracted from infected cell cultures by a modified Hirt procedure and analysed in neutral sucrose gradients (Carter *et al.*, 1979).

Purification and analysis of virus particles. Infected cells were harvested by shaking from the flask, washed twice in ice-cold phosphate buffered saline (PBS), resuspended in TE buffer (50 mM Tris-HCl (pH 8.7), 0.5 mM EDTA) and frozen at –20°. After thawing, the cells were sonicated for 1 min in a Branson Sonifier using the microtip attachment at setting 4. *n*-Butanol was then added to a final concentration of 1% and the lysate was incubated at 4° for 1 hr. CsCl and additional TE buffer were added to a volume of 12.2 ml and a refractive index of 1.3710 (density 1.3918 g/cm³). Samples were centrifuged in an SW-41 rotor at 15° for 48–65 hr at 30,000–39,000 rpm. The prolonged period of centrifugation was required in order to pellet all the free DNA contained in the sample.

For quantitative analysis of AAV particles from all regions of the CsCl equilibrium gradient the following contiguous regions were pooled: density 1.45 (1.450–1.435), 1.41 (1.395–1.435), 1.37 (1.365–1.390), 1.35 (1.340–1.360), and 1.32 (1.310–1.338). There were no additional AAV particles lighter than this density. The pooled samples were then dialyzed for 1 hr against TE buffer containing 0.3 M NaCl. Aliquots (usually 300 μ l) of each sample were applied to a 15–30% sucrose gradient prepared in TE buffer containing 1 M NaCl and centrifuged at 35,000 rpm for either 120 or 165 min at 15° in the SW 41 rotor. Virus particle yields were quantitated by computing the radioactivity recovered in individual components in the velocity sedimentation. The recovered radioactivity was then normalized to the volume of the pooled fractions of the CsCl equilibrium gradient.

DNase sensitivity of viral particles. Virus particles obtained from CsCl or sucrose gradients were dialyzed for 18 hr against a buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.3 M NaCl. MgCl₂

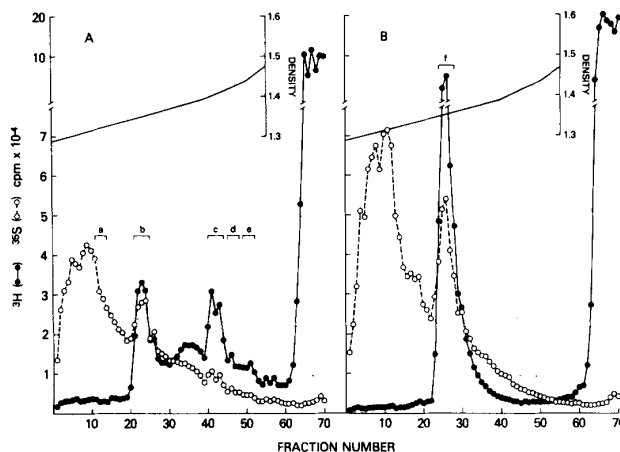


FIG. 1. CsCl banding profile of lysates from continuously labeled cultures. (A) AAV + Ad5wt. (B) Ad5wt alone. [^3H]Thymidine (● — ●), [^{35}S]methionine (○ — ○).

was added to a final concentration of 20 mM and samples were incubated for 30 min at 37° with 24 $\mu\text{g}/\text{ml}$ pancreatic DNase 1 (Worthington Biochemicals Corp.) then analyzed by velocity sedimentation in sucrose gradients.

RESULTS

Ultracentrifugal Properties of AAV Particles from Infected Cells

Preliminary experiments showed that most of the AAV particles accumulated between 12 and 30 hr after infection. Infected cultures were labeled continuously with both [^3H]thymidine and [^{35}S]methionine from 18–30 hr after infection. In cells infected with H5wt alone (Fig. 1B), the only species labeled in both DNA and protein was the mature adenovirus banding at 1.35 g/cm^3 (pool f). In the presence of AAV (Fig. 1A), the yield of adenovirus particles (pool b) was significantly depressed and several AAV components were present. Regions of the CsCl gradients were pooled and resedimented in neutral sucrose velocity gradients (Fig. 2).

The 1.32 g/cm^3 region (pool a, Figs. 1 and 2) contained a 66 S AAV component having an extremely low DNA/protein ratio which corresponded to "empty" AAV capsids. The AAV(1.35) particles (pool b, Figs. 1 and 2)

had a higher sedimentation coefficient (75 S) and an increased DNA/protein ratio corresponding to those particles containing DI genomes about one-fifth the size of the normal AAV DNA. The AAV(1.37) component (fractions 30–40, Fig. 1A) was not separately analyzed in this experiment but also comprises mainly AAV DI particles (see below). The AAV(1.41) particles (pool c, Figs. 1 and 2) contained the 110 S infectious component and there was some contamination with the larger DI particles shown by the slowly sedimenting shoulder at about 92 S. The AAV(1.43) (pool d) and AAV(1.45) (pool e) components were full particles with a DNA/protein ratio similar to AAV(1.41) particles.

In the pool of AAV(1.45) particles (pool e, Figs. 1 and 2) there was a second, clearly discernible component, AAV(SP), which sedimented at about 60 S but had a DNA/protein ratio similar to that of the 110 S particles. The density of this component ranged from 1.41 to 1.45 g/cm^3 .

All of the pooled regions from the CsCl gradients contained some amount of ^3H or ^{35}S label which remained at the top of the sucrose gradients (Figs. 2a–f; fractions 45–50). All of the adenovirus components with a buoyant density greater than 1.29 g/cm^3 , including mature adenovirions (Figs. 2b and f) and empty virions (Fig. 2a), sedimented onto the CsCl cushion at the bottom of the sucrose gradients (Fig. 2, fractions 6–10).

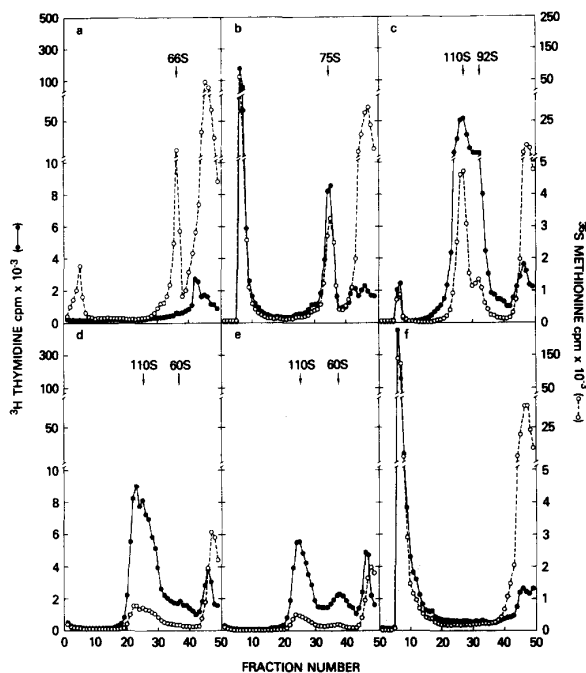


FIG. 2. Velocity sedimentation of virus particles from the regions designated (a) to (f) in Fig. 1. The average density of each pooled regions, respectively, was (a) 1.32, (b) 1.35, (c) 1.41, (d) 1.43, (e) 1.45, and (f) 1.35.

Analysis of AAV Particles Labeled in Pulse-Chase Conditions

In all the subsequent experiments to study the kinetics of AAV assembly cells were infected with AAV and the mutant helper ts215 and grown at 40° then pulse-labeled with [³⁵S]methionine beginning at 18 hr after infection. One example of the data obtained, from a culture that was pulsed for 10 min and chased for 4 hr, is shown in Fig. 3. From the CsCl gradient of the lysate (Fig. 3a), contiguous regions of the gradient were pooled and analyzed further by sedimentation in neutral sucrose gradients (panels b–f). In this experiment the AAV(1.37) particles were also analyzed (panel d) and represent a population of 82 S DI particles. Division of the CsCl gradient into contiguous density regions allowed identification of all the AAV particle species present. The components observed in the pulse-chase (Fig. 3) are similar to those observed in the continuous label (Figs. 1

and 2) except that the amount of AAV(SP) particles (Figs. 3e and f, fractions 30–32) was much less predominant following the chase.

Figure 4 shows the results of a pulse-chase experiment using [³⁵S]methionine. The most rapidly labeled components were AAV(1.32) and (1.35) particles, both of which obtained maximum radioactivity levels by 10 to 20 min after the pulse. Subsequently, the amount of label in AAV(1.32) particles decreased by about 30% whereas that in 1.35 particles remained constant. AAV(SP) and AAV(1.37) both began to accumulate label soon after the pulse but at a slower rate than AAV(1.32) or AAV(1.35). At later times in the chase the amount of labeled AAV(SP) decreased. The most slowly labeled components were AAV(1.41) and AAV(1.45) which began to accumulate label from about 20 min after the pulse.

The loss of radioactive label from AAV(1.32) during the chase (Fig. 4) was not entirely accounted for by increased incorpora-

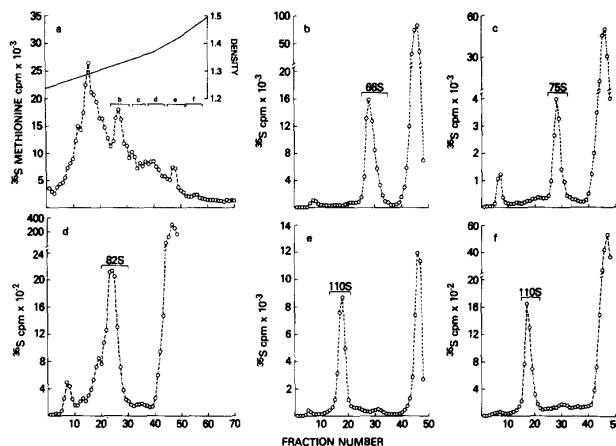


FIG. 3. Pulse-chase labeling of AAV particles. Cells were infected with AAV and Ad2ts215 at 40°. At 18 hr after infection, the culture was pulse-labeled for 10 min with [^{35}S]methionine, then chased for 4 hr. The culture was then lysed and centrifuged to equilibrium in a CsCl density gradient (a). Regions indicated in panel a were pooled and analyzed by velocity sedimentation in sucrose gradients: 1.32 (b), 1.35 (c), 1.37 (d), 1.41 (e), and 1.45 (f).

tion in the other particles (1.41, 1.45, 1.37). In a similar experiment using a longer chase (Fig. 5), the decrease in the amount of ^{35}S label in AAV(1.32) was quantitatively accounted for by the increased labeling of the other components. The failure to observe a quantitative shift in labeling at earlier times in the chase (Fig. 4) may indicate instability of some assembly intermediates, e.g., AAV(SP) as discussed below.

To study the incorporation of AAV DNA into mature particles a similar pulse-chase experiment was performed using [^3H]thymidine (Fig. 6). The number of particles is not proportional to the amount of DNA label because AAV(1.37) and (1.35) particles have smaller genomes than AAV(1.41) and (1.45) particles. The order of the labeling of the various density species of AAV with [^3H]thymidine (Fig. 6) is consistent with that observed with [^{35}S]methionine (Figs. 4, 5) except that AAV(1.32) particles were labeled only slowly after the pulse and to a very low level. This reflects the fact that the smallest DI genomes are packaged into virions having a density indistinguishable from that of empty 1.32 virions. This may also account for the apparent failure to chase all the ^{35}S label from 1.32 particles.

The AAV(1.35) particles were labeled

more slowly with [^3H]thymidine than with [^{35}S]methionine (cf. Figs. 4, 5, and 6) and like all other DNA-containing species had no ^3H label immediately at the end of the pulse (30 min). Also, there was an increase in sedimentation velocity (from 66 to 75 S) of ^{35}S -labeled AAV(1.35) particles during the chase, concomitant with the appearance of ^3H label in this component (data not shown). This suggests that the AAV(1.35) particles seen in Figs. 4 and 5 may be different species early and late in the chase representing empty particles and DNA-containing DI particles, respectively. The "early" AAV(1.35) particles with apparently no DNA may represent either contamination from the AAV(1.32) region in the CsCl gradient or an alternative structure without DNA that bands in CsCl at 1.35 g/cm ^{3} . Further experiments are required to elucidate this point.

The experiments described thus far suggest that empty AAV(1.32) particles are precursors of DNA-containing full or DI particles and that AAV(SP) may be an intermediate in this process. Whether full AAV-(1.45) particles are precursors to full AAV-(1.41) particles is unclear. In additional experiments (not shown) using longer pulse periods (up to 2 hr) [^3H]thymidine label con-

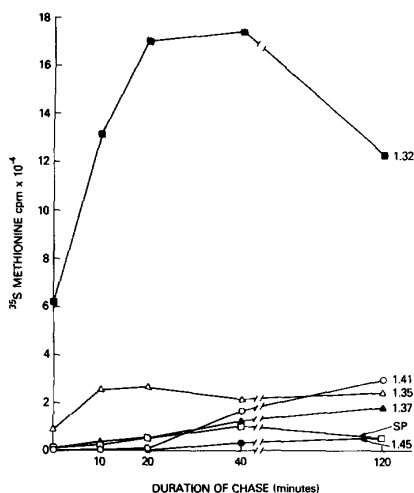


FIG. 4. Pulse-chase kinetics of AAV particles with [^{35}S]methionine. KB cells infected with AAV and Ad2ts215 at 40° were labeled for 10 min with [^{35}S]methionine at 18 hr after infection ($50\ \mu\text{Ci}/\text{ml}$) then chased. The AAV (1.32), (1.35), (1.37), (1.41), and (1.45) classes correspond to the 66, 75, 82, 110, and 110 S species, respectively, shown in Fig. 6. The AAV-(SP) was summed from both the 1.41 and 1.45 density regions.

tinued to accumulate in AAV(1.41), but not AAV(1.45) particles, throughout the chase period, but there was no preferential labeling of AAV(1.45) during the pulse. In contrast, the AAV(SP) particles incorporated label initially more rapidly than either 1.41 or 1.45 particles but the relative amount of AAV(SP) decreased progressively throughout the pulse and chase. Again these data suggest that AAV(SP) may be a precursor of 1.41 or 1.45 particles or both.

The data however do not clearly show a precursor-product relationship between AAV(1.45) and AAV(1.41).

Effect of Inhibitors on AAV Replication

L-Canavanine was reported to inhibit formation of the 60K polypeptide C, the major component (by mass) of mature AAV particles (Buller and Rose, 1978). We therefore examined the effect of L-canavanine and of the DNA synthesis inhibitor hydroxyurea on AAV particle formation and DNA syn-

thesis. One set of cultures was analyzed for virus particles by banding in CsCl and subsequent velocity sedimentation (Table 1). A second set of cultures was analyzed for viral DNA synthesis by Hirt fractionation (data not shown).

Hydroxyurea, at a concentration of $500\ \mu\text{g}/\text{ml}$, almost completely inhibited labeling of virus DNA whereas $10\ \text{mM}$ canavanine had no apparent effect. At $50\ \mu\text{g}/\text{ml}$, hydroxyurea inhibited AAV DNA synthesis by 60–70%. Although canavanine treatment had no effect on labeling of AAV DNA it decreased the labeling of AAV particles (Table 1). The greatest decrease was in AAV(1.41) particles but there was also a significant decrease in AAV(1.32) particles. Thus, canavanine prevented formation of both empty and full virions of AAV. That the full virions were depressed even further than the empty virions suggests that many of the empty virions made in the presence of canavanine contained aberrant proteins and thus would not be used for assembly.

Additional support for the role of AAV-(1.32) empty capsids as precursors to mature AAV virions was provided by the results with hydroxyurea (Table 1). The labeling of AAV(1.41) and (1.37) particles was inhibited but there was increased accumulation of AAV(1.32) empty capsids.

As shown in Table 1, hydroxyurea re-

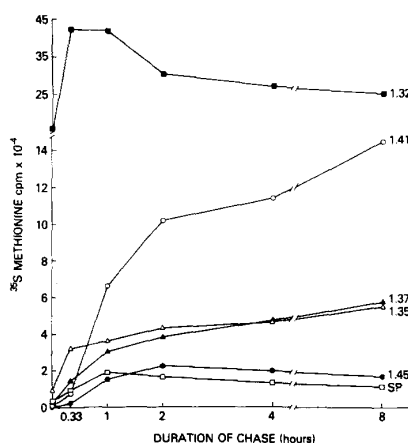


FIG. 5. Pulse-chase kinetics of AAV particles with [^{35}S]methionine. The conditions were as in Fig. 7.

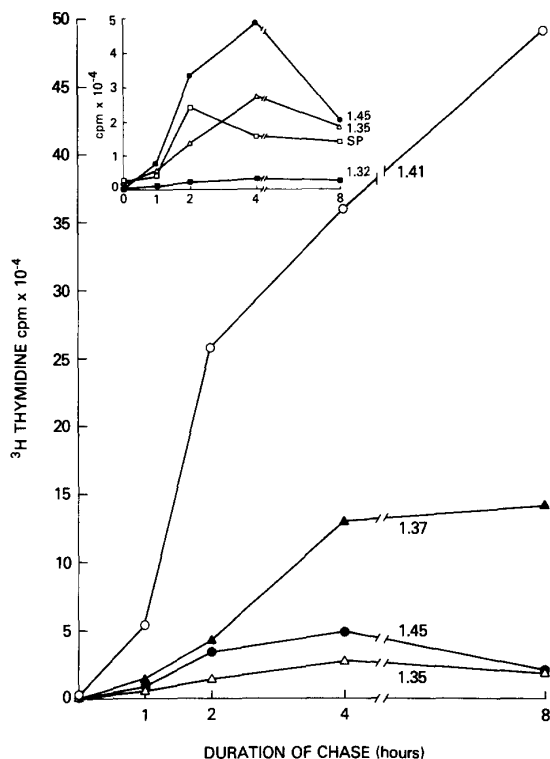


FIG. 6. Pulse-chase kinetics of AAV particles with [^3H]thymidine label. KB cells infected with AAV and Ad2ts215 at 40° . At 18 hr after infection, the cultures were labeled for 30 min with [^3H]thymidine then chased. An expanded scale is presented in the inset.

duced the yield of ^3H -labeled AAV(1.35) whereas ^{35}S -labeled AAV(1.35) was equivalent to the control. Also, the sedimentation rate of AAV(1.35) was 75 S in the control experiment but the (1.35) particles assembled in the presence of hydroxyurea sedimented with AAV(1.32) at 66 S (not shown). These observations are consistent with those discussed above that there may be two AAV components which band in CsCl at 1.35 g/cm^3 .

Effect of L-Canavanine on Pulse-Chase Kinetics

The effect of L-canavanine on pulse-chase kinetics of AAV assembly was examined. Figure 7 illustrates the results obtained for AAV(1.41) particles, but analogous results were obtained for the other classes of AAV

particles. When canavanine was present during only the chase (Fig. 7) the labeling of AAV(1.41) particles with [^3H]thymidine was progressively depressed with time during the chase.

An even greater reduction in ^3H labeling of AAV(1.41) particles was observed when canavanine was present during the 10-min pulse as well as the chase (Fig. 7). That the additional short period of canavanine treatment has such a significant effect suggests that the pool of precursor proteins used for encapsidation can be depleted rapidly.

In contrast, labeling of AAV(1.41) particles with [^{35}S]methionine was not depressed greatly by the presence of canavanine during the chase. Thus, the ^3H : ^{35}S ratio for particles synthesized in the presence of canavanine was lower than for the control

TABLE 1

EFFECT OF PRETREATMENT WITH L-CANAVANINE OR HYDROXYUREA ON AAV PARTICLE ASSEMBLY^a

AAV particle type	Total radioactivity (cpm) in AAV particles after pretreatment of cultures of cultures with							
	No addition		L-Canavanine (10 mM)		Hydroxyurea			
					500 μ g/ml		50 μ g/ml	
	³⁵ S	³ H	³⁵ S	³ H	³⁵ S	³ H	³⁵ S	³ H
AAV(1.32) ^b	300,200	—	63,522 (21)	—	791,538 (263)	—	685,902 (228)	—
AAV(1.35)	77,277	24,211	14,656 (19)	8,391 (35)	80,065 (104)	3,999 (17)	93,138 (120)	15,456 (64)
AAV(1.37)	77,151	68,765	4,299 (6)	9,116 (13)	27,666 (36)	4,182 (6)	28,635 (37)	16,262 (24)
AAV(1.41)	139,518	241,297	399 (0.3)	7,296 (3)	17,535 (12)	4,016 (2)	19,133 (14)	22,680 (9)
AAV(1.45)	18,370	NT ^c	NT	NT	NT	NT	3,222 (18)	3,929

^a KB cells were infected with AAV and Ad2ts215 at 40°. At 17 hr after infection, the medium was removed and fresh medium was added which contained either L-canavanine (10 mM) or hydroxyurea (500 μ g/ml and 50 μ g/ml). After 1 hr, [³H]thymidine and [³⁵S]methionine were added and incubation was continued for 4 hr. Infected cell lysates were then analyzed for virus particles or for virus DNA synthesis (see text). Numbers in parentheses indicate the amount of radioactivity as a percentage of the amount of the same isotope in the same particle type in the control culture.

^b For the ³H label in AAV(1.32) the radioactivity was too low to measure reliably.

^c Not tested.

particles. This indicates more efficient utilization of the previously synthesized, ³⁵S-labeled, AAV protein when the chase included canavanine. We interpret this to mean that canavanine inhibits assembly by causing production of new aberrant AAV proteins rather than by interfering with the use of the pool of previously synthesized AAV protein.

Effect of Hydroxyurea on Pulse-Chase Kinetics

To determine if ongoing DNA replication was necessary to package viral DNA we studied the effect of hydroxyurea on the pulse-chase kinetics of assembly. In the conditions used viral DNA synthesis was inhibited within 15 min of addition of hydroxyurea (data not shown). In the pulse-chase experiment (Fig. 8) the [³⁵S]methionine incorporation showed a significant re-

duction (36%) in assembly of AAV(1.41) particles as early as 20 min after the end of the pulse. A similar decrease was seen in AAV(SP) at 20 min. More importantly, the inhibition of particle formation relative to the control then remained constant throughout the entire 4-hr chase period. This is in distinct contrast to the observed labeling kinetics when a pulse label was chased in the presence of canavanine (Fig. 7). A similar observation was made for AAV(1.37) and AAV(1.45) particles. However, there was accumulation of ³⁵S label in AAV(1.32) particles which is consistent with the hydroxyurea pretreatment experiment (Table 1).

In the experiment of Fig. 8, the degree of inhibition of [³H]thymidine incorporation by hydroxyurea (not shown) was very similar to that shown with protein label (Fig. 8). Furthermore, the ³H DNA:³⁵S protein ratios of all particles (except AAV(1.32) where

^{35}S empty particles accumulated) were similar in both the control and hydroxyurea samples. These results show that accumulation of DNA-containing AAV particles could occur in the absence of DNA synthesis though in a reduced amount. The fact that there was no change in the ^3H : ^{35}S ratio of the (1.41) particles (i.e., that presynthesized [^3H]DNA was not utilized more efficiently during the hydroxyurea chase), in contrast to that seen in the canavanine chase (Fig. 7), suggests the absence of an excess pool of free single strands.

Characterization of AAV(SP)

Since AAV(SP) may be an assembly intermediate we characterized its physical structure further. Figure 9 shows the alkaline sucrose sedimentation profile of DNA from AAV(SP) particles. The majority of the [^3H]DNA was genome length but about 20% of the material sedimented more slowly. This suggests that the DNA contains both full-length genomes and deleted DI genomes. This has been confirmed by restriction endonuclease cleavage of this DNA (L. M. de la Maza and B. J. Carter, unpublished).

The DNase sensitivity of AAV(SP) particles was examined in separate preparations that were labeled with either [^{35}S]methionine for 10 min or [^3H]thymidine for 4 hr. The ^3H -labeled material was obtained from the 60 S peak of a velocity sedimentation of AAV(1.41). This material, on resedimentation (Fig. 10A), was shown to contain some 110 S particles (26% of total). These 110 S particles (Fig. 10) were unaffected by DNase treatment, while 82% of the ^3H label in the 60 S AAV(SP) component was digested and accumulated at the top of the gradient. The residual, DNase-resistant [^3H]thymidine-labeled particles from AAV-(SP) had a sedimentation rate of 75 S.

[^{35}S]Methionine-labeled particles were tested for DNase sensitivity directly from a CsCl AAV(1.41) pool (Fig. 10B). The ^{35}S label in both the 110 S particles and the 60 S AAV(SP) was resistant to DNase. After the DNase digestion the sedimentation rate of AAV(SP) increased to 66 S which is the same value as empty AAV(1.32) particles. These results suggest that AAV-

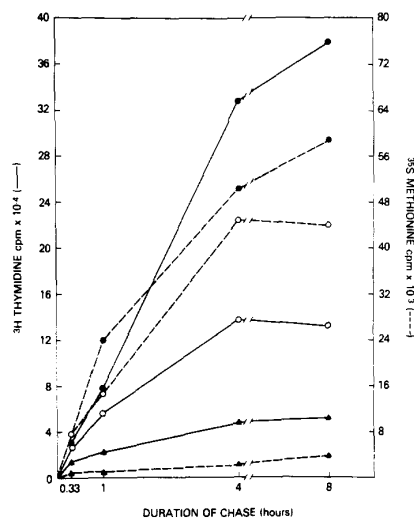


FIG. 7. Effect of L-canavanine on pulse-chase kinetics of AAV(1.41) particles. KB cells infected with AAV and Ad2ts215 at 40° were pulse-labeled, at 18 hr after infection, for 10 min with [^3H]thymidine and [^{35}S]methionine then chased. Solid lines, [^3H]thymidine; broken lines, [^{35}S]methionine; (●) control (no canavanine), (○) L-canavanine (10 mM) present during the chase only, (▲) L-canavanine (10 mM) present during the pulse and chase.

(SP) may represent an empty capsid associated with AAV DNA molecules in a structure which has a more open conformation than that of mature particles. The DNase-resistant ^3H -labeled particles may represent either DNA that is partially protected by its position within the precursor structure or contaminating mature DI particles in which the entire molecule is resistant to DNase. The ^3H or ^{35}S label in the major peaks of AAV(1.45), AAV(1.37), and AAV(1.35) were also completely resistant to DNase digestion.

DISCUSSION

The data described here are consistent with a general model for AAV assembly. In this model DNA progeny strands are supplied by strand displacement replication from duplex replicating intermediates (RI) as shown by others (Strauss *et al.*, 1976; Tattersall *et al.*, 1973; Rhode, 1978; Siegl and Gautschi, 1976; Gunther and May, 1976).

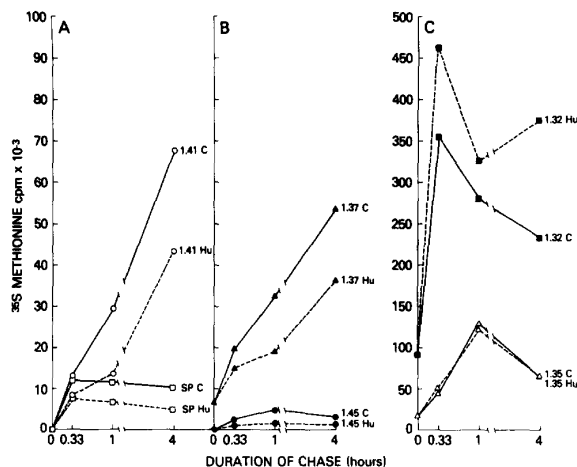


FIG. 8. Effect of hydroxyurea on pulse-chase kinetics. KB cells infected with AAV and Ad2ts215 at 40° were pulse-labeled at 18 hr for 10 min with [^3H]thymidine and [^{35}S]methionine then chased. Solid lines, control (C). Broken lines, hydroxyurea (500 $\mu\text{g}/\text{ml}$) present during the chase (Hu). Only the data for the [^{35}S]methionine label are shown.

Newly synthesized AAV protein is rapidly assembled into empty 1.32 capsids which then associate with progeny DNA strands concomitant with, or soon after, displacement to form intermediate structures. These intermediate structures are converted

slowly over several hours to give mature “full” particles containing either a standard or DI genome. DNA replication can occur in the absence of capsid assembly. Although DNA replication is presumably required to provide the progeny strands, the virus “maturation” process occurs in the absence of DNA synthesis.

The role of the empty 1.32 particles as precursors of full particles is suggested by the pulse-chase labeling kinetics. Also, prior inhibition of DNA synthesis with hydroxyurea caused increased accumulation of empty particles and inhibited the appearance of full particles. This observation also indicated that empty particles are not derived from disruption of mature particles. Addition of L-canavanine inhibited formation of both empty and full particles. This observation can be accounted for by inhibition of production of the 60K (VP3) major capsid protein of AAV (Buller and Rose, 1978).

The empty particles accumulate rapidly in 10 to 20 min following a short radioactive pulse label but mature “full” virions accumulated slowly over 4 to 8 hr. The slow accumulation of progeny DNA strands into full particles appears to reflect an intrinsic property of the maturation process rather

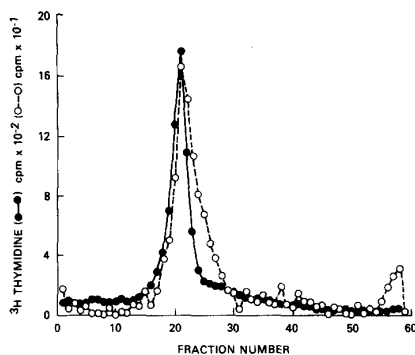


FIG. 9. Analysis of AAV(SP) DNA. An infected culture was labeled with [^3H]thymidine from 18–24 hr after infection. The 1.41 g/cm^3 density region of the CsCl equilibrium gradient was sedimented on a neutral sucrose velocity gradient. The 110 S AAV(1.41) and 60 S AAV(SP) peaks were pooled separately, dialyzed, and sedimented in parallel 5–20% alkaline sucrose gradients at 33,000 rpm for 21.5 hr at 4° in the SW 41 Beckman rotor. (—●—●) 110 S particle, AAV(1.41) DNA. (---○---○) 60 S particle, AAV(SP) DNA.

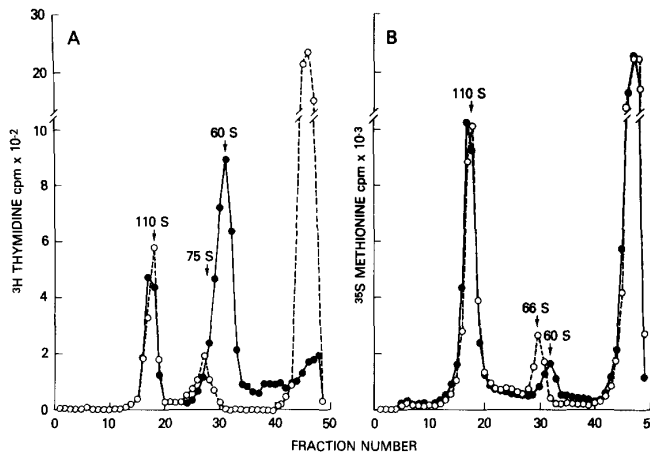


FIG. 10. DNase sensitivity of AAV(SP). (A) ^3H -labeled 60 S AAV(SP) was treated with DNase and sedimented on 15–30% sucrose neutral gradients. (● — ●) AAV(SP) incubated without DNase; (○ — ○) incubated with DNase. (B) ^{35}S -Labeled AAV(1.41) particles obtained from a culture pulsed for 10 min and chased for 1 hr were treated with DNase and resedimented on 15–30% neutral sucrose gradients. (● — ●) Control incubated without DNase; (○ — ○) incubated with DNase.

than delayed exit from, or reutilization in, the replicating pool. Several arguments support this suggestion. Inhibition of DNA synthesis with hydroxyurea immediately after a pulse decreased encapsidation of the pulse-labeled DNA by 35% only, but more importantly the kinetics of accumulation were not altered. The decrease of 35% presumably reflects the proportion of pulse-labeled DNA that was not displaced before hydroxyurea inhibition. However, as noted above, addition of hydroxyurea 1 hr prior to labeling with ^{35}S methionine almost completely inhibited assembly of full particles. These observations together suggest that there is no pool of free progeny DNA strands and that the single DNA strands may be committed to encapsidation, concomitantly with, or soon after, synthesis. This “commitment” may involve association of the displaced progeny DNA strands with a precursor empty capsid to form an intermediate structure.

The AAV(SP) component identified in these experiments appears to have both the kinetic and physical properties of the assembly intermediate suggested above. This component is probably not produced merely from aggregation of free DNA and empty capsids because of its labeling kinetics and

its relative stability in CsCl. Also, it does not appear to arise from disruption of AAV(1.41) particles. AAV(1.41) particles are completely stable to treatment with trypsin, DNase, deoxycholate, EDTA, heating at 60° , or incubation with infected cell lysates in either the trypsin-deoxycholate or butanol isolation procedures. Although the possibility is not excluded, we have been unable to obtain convincing evidence of a precursor-product relationship between 1.45 and 1.41 particles either in this study or other experiments using ^{32}P as the pulse label and the trypsin-deoxycholate purification procedure (L. M. de la Maza and B. J. Carter, unpublished). The small decrease in 1.45 particles late after a pulse may indicate that this is a precursor of 1.41 particles. However, AAV(1.45) could also be an alternative end product of assembly.

It has been suggested that displacement synthesis of parvovirus progeny strands may be driven by concomitant packaging (Tattersall and Ward, 1976). This is not inconsistent with the model proposed above except that the concomitant packaging may involve association with the empty capsid in an intermediate structure rather than immediate assembly into a mature stable virion. However, we have shown that at least the

subsequent "maturation" of AAV particles still occurred when DNA synthesis was blocked with hydroxyurea. Also, AAV DNA synthesis continued in the presence of L-canavanine which inhibited assembly of both empty and full particles. Thus, AAV DNA replication and virion maturation are not directly linked. However, for AAV it is difficult to readily distinguish progeny strand replication from RI replication because of reannealing of the plus and minus complementary strands. Thus synthesis of progeny strands but not RIs of AAV DNA might be directly linked to presence of capsids. Preliminary experiments support this idea (M. W. Myers and B. J. Carter, unpublished).

The mode of assembly described here for AAV is consistent with the apparent absence of any internal nucleoprotein core structure in parvoviruses in contrast to those present in other DNA-containing viruses including papova-, adeno-, herpes-, and poxviruses. Also, individual AAV DI genomes containing deletions of up to 90% of genome length are packaged into AAV particles having an apparently normal capsid (de la Maza and Carter, 1978; F. T. Jay, L. M. de la Maza, and B. J. Carter, unpublished). Thus, DNA is not apparently required for the assembly or structural integrity of the AAV protein capsid.

REFERENCES

- BULLER, R. M. L., and ROSE, J. A. (1978). Characterization of adenovirus-associated virus-induced polypeptides in KB cells. *J. Virol.* **25**, 331-338.
- CARTER, B. J., LAUGHLIN, C. A., DE LA MAZA, L. M., and MYERS, M. (1979). Adeno-associated virus auto-interference. *Virology* **92**, 449-462.
- DE LA MAZA, L. M., and CARTER, B. J. (1978). DNA structure of incomplete adeno-associated virus particles. In "Replication of Mammalian Parvoviruses" (D. C. Ward and P. Tattersall, eds.), pp. 193-204. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- GUNTHER, M., and MAY, P. (1976). Isolation and characterization of monomeric and dimeric forms of replication intermediates of Kilham rat virus DNA. *J. Virol.* **20**, 86-95.
- LAUGHLIN, C. A., MYERS, M. W., RISIN, D. L., and CARTER, B. J. (1979). Defective-interfering particles of the human parvovirus adeno-associated virus. *Virology* **94**, 162-174.
- RHODE, S. L. (1978). H-1 DNA synthesis. In "Replication of Mammalian Parvoviruses" (D. C. Ward and P. Tattersall, eds.), pp. 279-296. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- ROSE, J. A., BERNIS, K. I., HOGGAN, M. D., and KOCZOT, F. J. (1969). Evidence for a single-stranded adenovirus-associated virus genome: Formation of a DNA density hybrid on release of viral DNA. *Proc. Nat. Acad. Sci. USA* **64**, 863-869.
- SIEGL, G., and GAUTSCHI, M. (1976). Multiplication of parvovirus Lu III in a synchronized culture system III. Replication of viral DNA. *J. Virol.* **17**, 841-853.
- STRAUSS, S. W., SEBRING, E. D., and ROSE, J. A. (1976). Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. *Proc. Nat. Acad. Sci. USA* **73**, 742-746.
- TATTERSALL, P., and WARD, D. C. (1976). Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. *Nature (London)* **263**, 106-109.
- TATTERSALL, P., CRAWFORD, L. V., and SHATKIN, A. J. (1973). Replication of the parvovirus MVM. II. Isolation and characterization of intermediates in the replication of the viral deoxyribonucleic acid. *J. Virol.* **12**, 1446-1456.