Adeno-associated Virus Replication

THE EFFECT OF L-CANAVANINE OR A HELPER VIRUS MUTATION ON ACCUMULATION OF VIRAL CAPSIDS AND PROGENY SINGLE-STRANDED DNA*

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Maureen W. Myers and Barrie J. Carter
From the Laboratory of Experimental Pathology, National Institute of Arthritis Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

We have studied the relationship between adeno-associated virus (AAV) DNA replication and virus particle assembly. Formation of empty or full particles and accumulation of AAV capsid proteins was prevented in the presence of the arginine analogue, L-canavanine, or when a temperature-sensitive helper adenovirus was used at the nonpermissive temperature. In each case there was a concomitant inhibition of AAV single-stranded (progeny) DNA accumulation but little or no effect upon synthesis of AAV duplex, replicating form DNA. These results indicate that AAV protein, perhaps in the form of assembled capsids, is required for AAV single-stranded progeny DNA accumulation.

Parvoviruses are eukaryotic viruses that contain linear single-stranded DNA genomes (about 4700 nucleotides in length) having palindromic sequences of 100 to 200 nucleotides in length at their 5' and 3' termini. The autonomous (nondetective) parvoviruses such as the minute virus of mice or the hamster virus, H1, require certain host cell functions expressed only in S phase of the cell cycle in order to replicate. These parvoviruses package DNA strands of only one polarity and the 5' and 3' palindromic sequences are different. The defective parvoviruses consist of the adeno-associated viruses which grow only in cells coinfected with an adenovirus or a herpesvirus. The defective viruses package strands of either polarity (plus or minus) into separate particles. The isolated plus and minus strands can anneal to form duplex DNA. In addition, the 5'- and 3'-terminal palindromes of AAV DNA are identical. Parvoviruses are extensively reviewed in Reference 1.

Replication of parvovirus DNA is postulated to occur by a self-priming mechanism (2, 3). This yields a duplex RF DNA molecule of monomer length in which the daughter and parental DNA strands are covalently joined. The terminal hairpin structure may be converted to a normal DNA duplex structure by a Cavalier-Smith mechanism (4) in which the parental palindrome is transferred to the daughter strand and a new palindromic sequence is generated on the parental strand by a DNA repair process. Additional rounds of DNA synthesis form oligomeric RF molecules or produce progeny SS DNA apparently by a strand displacement mechanism.

Studies of intracellular parvovirus DNA replication have provided evidence for monomeric and oligomeric duplex RF DNA molecules, some of which contain hairpin termini and covalently joined parental (template) and daughter strands (2, 3, 5). The mechanism of progeny SS DNA synthesis and the relationship of this to encapsidation of the DNA is much less clear. It has been suggested that these two events may be linked in a concerted mechanism which would provide the energy required for strand displacement and concomitant encapsidation of the displaced strand (3).

Studies of AAV variants, containing internal deletions in the DNA genome (6, 7), implied that an AAV protein is required for DNA synthesis. More recent studies of AAV assembly indicated that AAV DNA is packaged into a preformed capsid and that association of the SS DNA with an empty capsid appeared to occur rapidly, perhaps concomitantly with synthesis of the strand (8). In the presence of L-canavanine which prevented synthesis of the major AAV capsid protein (9), AAV DNA synthesis still occurred (8). Also, when an adenovirus having a temperature-sensitive mutation in the adenovirus early region 2 gene was used as a helper at the nonpermissive temperature, all of the AAV capsid proteins failed to accumulate but AAV DNA replication occurred (10). However, in the conditions used in these studies synthesis of AAV RF DNA and SS DNA were not distinguished.

We show here that inhibition of AAV capsid accumulation was accompanied by inhibition of AAV SS DNA, but not RF DNA, accumulation. These observations suggest that accumulation of AAV SS DNA is linked to accumulation of AAV capsid proteins. The experiments described provide independent procedures to experimentally discriminate AAV SS DNA synthesis from that of RF synthesis.

EXPERIMENTAL PROCEDURES

Viruses and Cells—Stocks of adeno-associated virus type 2 or adenovirus were grown and assayed as described before (7, 8, 11). The AAV inoculum was either a crude cell lysate or the purified infectious particles (density of 1.41 g/cm3 in CsCl) obtained by banding to equilibrium three times in CsCl gradients (11). In the experiments reported here either inoculum gave similar results. The Ad5 tol25 mutant and the parent wild type strain were those described by Ensinger and Ginsberg (12). For all the experiments described here, monolayers of KB cells (75 cm2) grown in Eagle's minimal essential medium supplemented with Earle's salts and 5% fetal calf serum (GIBCO Laboratories, Grand Island, NY) were infected with adenovirus and AAV as described before (7, 8, 11).

Analysis of Intracellular Virus DNA—[Methyl-3H]thymidine (specific activity, 40 to 60 Ci/mm; New England Nuclear) was added to the culture medium to a final concentration of 100 μCi/ml. Viral DNA was selectively extracted by the modified Hirt procedure exactly as described before (11) except that proteinase K (fungal, 3.4214, EM Biochemicals, Elmsford, NY) was added at a final concentration of 20 μg/ml together with the pronase. Also the DNA-containing supernatant was not reannealed but was dialyzed at 2°C against 2 changes of 1000 volumes of buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM Na2EDTA. DNA was then analyzed by velocity sedimentation at 2°C in neutral 5 to 20% sucrose gradients (12 ml) containing 1 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM Na2EDTA, and 0.15% Sarkosyl. Gradients were centrifuged in nitrocellulose tubes

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1 The abbreviations used are: AAV, adeno-associated virus; SS DNA, single-stranded DNA; RF DNA, replicating form DNA.

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in the SW41 rotor of a Beckman ultracentrifuge at 30,000 rpm for 16 h. Under these conditions, single strands of AAV DNA did not renatate.

Analysis of AAV Particle Production—Production of AAV particles was measured exactly as described before (8). Briefly, cell cultures were incubated in medium containing one-tenth the normal concentration of methionine, [35S]methionine (specific activity, 900 to 1100 Ci/mmol; New England Nuclear) at 100 μCi/ml (final concentration) and [methyl-3H]thyminide at 100 μCi/ml (final concentration). Cells were infected with 100 units of virus per cell and incubated with 1-butanol then banded to equilibrium in CsCl gradients. Particles from various regions of the CsCl gradient were then analysed by velocity sedimentation in neutral sucrose gradients. Full AAV particles were measured as particles with a density in CsCl of 1.41 g/cm3 and a sedimentation constant of 66 S. Full particles were quantitated using incorporation of [3H]thymidine ([35S]methionine label which gave similar results), whereas empty particles were quantitated using [35S]methionine incorporation.

RESULTS AND DISCUSSION

The arginine analogue, L-canavanine inhibits accumulation of both the AAV 60,000 (C or VP3) protein which is the major protein component (approximately 85% by mass) of empty or full AAV capsids and a nonstructural 24,000 polypeptide (9). In contrast, accumulation of the two minor components of the AAV capsid, the 72,000 (B or VP2) and 85,000 (A or VP1) proteins, is not affected. The drug apparently inhibits a post-translational cleavage required to produce the 60,000 VP3.

We investigated the effect of L-canavanine on AAV SS DNA synthesis and AAV particle formation. Cells were infected with wild type adenovirus 5 and then superinfected 9 h later with AAV2. Under these conditions, AAV DNA and RNA synthesis begin about 3 to 4 h after infection with AAV and assembly of AAV particles is essentially complete by 18 to 20 h after infection with AAV (13). Accordingly, the infected cultures were incubated from 4 to 18 h after addition of AAV with [3H]thymidine to label viral DNA or jointly with [3H]thymidine and [35S]methionine to label AAV particles.

DNA extracted from cells infected with wild type adenovirus alone contained a single 31 S species of mature adenovirus DNA duplexes whereas uninfected cells yielded no discrete, labeled DNA species (Fig. 1B). In the cells that were superinfected with AAV there were in the neutral sucrose gradient (Fig. 1A), at least four additional DNA species designated as 22 S, 18 S, 14.5 S, and 10 S, respectively. All four of these species represent AAV DNA as revealed by resedimentation of each in neutral and alkaline sucrose gradients (data not shown), by molecular hybridization to purified viral DNA (10), and by cleavage with specific endodeoxyribonucleases (11). The 22 S DNA represents AAV SS DNA. This DNA sedimented at 15 S in alkaline sucrose gradients but, when first incubated under annealing conditions, formed duplex molecules that sedimented at 14.5 S at neutral pH. These are characteristic properties of AAV SS DNA. The 14.5 S and 18 S DNAs in the gradients of Fig. 1A represent monomer and dimer duplex RF DNAs (2). Some of the 14.5 S monomer duplexes contained terminal cross-linked, (hairpinned) RF molecules and released dimer length single strands in alkaline sucrose (not shown). Similarly the 18 S dimer DNA in the neutral sucrose gradient (Fig. 1A) released, in alkaline conditions, a mixture of single strands of monomer, dimer, or tetramer length. The 10 S DNA (Fig. 1A) represents variant or defective-interfering AAV genomes. The properties and structures of these variant AAV genomes have been described elsewhere (6, 7, 11, 14).

Addition of L-canavanine 9 h after adenovirus infection (i.e. at the same time as the superinfection with AAV) had little apparent effect upon AAV 14.5 S and 18 S RF DNA synthesis but prevented accumulation of AAV 22 S SS DNA (Fig. 1A). The drug also effectively prevented synthesis or accumulation of both empty and full AAV particles (Table 1). Thus, the accumulation of AAV progeny SS DNA was correlated with the accumulation of capsids.

Growth of AAV in KB cells was defective when a mutant helper virus, Ad5 ts125, was used at the nonpermissive temperature (10). This mutant has a temperature-sensitive lesion in the adenovirus early region 2 gene for a 72,000 (E72) DNA-binding protein (12, 15–17). With the mutant helper, total AAV DNA synthesis was decreased not more than 2- to 4-fold, whereas AAV particle synthesis was decreased by 50- to 100-fold. Furthermore, the decrease in AAV particle synthesis appeared to be due to a decrease, of a similar order of magnitude, in the accumulation of all three of the AAV capsid proteins (10). We have therefore used the ts125 mutant as an independent method to inhibit AAV capsid protein accumulation and to assess the subsequent effect on AAV SS DNA accumulation.

Cells were simultaneously infected with AAV and a wild type or mutant helper and then grown at the nonpermissive temperature of 40°C. AAV DNA or particles were labeled as before but from 12 to 21 h after infection. This time represents, under the conditions of a simultaneous infection, the period of maximum rates of accumulation of AAV DNA and particles (8, 13). AAV particle production with the mutant helper virus was decreased at least 30-fold (Table II). Analysis of intracellular DNA (Fig. 2A) showed that with the mutant adenovirus helper there was a specific inhibition of accumulation of AAV 22 S SS DNA but only a very small effect on accumulation of 14.5 S or 18 S RF DNA. The temperature-sensitive mutation abolished all adenovirus DNA synthesis as shown by the absence of 31 S DNA (Fig. 2A). These results using the mutant helper virus are analogous to those described above using the metabolic analogue.

In the simultaneous infection experiment (Fig. 2), AAV infection resulted in an 8-fold inhibition of wild type adenovirus DNA replication as observed before (11), whereas in the preinfection protocol (Fig. 1), superinfection with AAV resulted in an inhibition of only 40%. Also as seen in Fig. 1B, addition of canavanine similarly decreased subsequent adenovirus DNA replication by about one-half. The combined effect upon adenovirus DNA replication of both L-canavanine and AAV superinfection appears to be directly additive (Fig. 1A). Similar effects on accumulation of AAV SS DNA and particles to those reported here were observed when L-canavanine was used in a simultaneous infection protocol or when the ts125 mutant was used in the preinfection protocol (data not shown).

The above experiments show that when accumulation of AAV capsid proteins and consequently AAV capsids was inhibited there was a concomitant failure to accumulate AAV SS DNA but little or no effect on AAV RF DNA synthesis. The simplest interpretation of our results is that one or more AAV proteins or the assembled AAV capsid is required to accumulate SS DNA. These results are analogous to those obtained with mutants of the autonomous parvovirus H1 having temperature-sensitive lesions in the viral capsid proteins (18). These mutants, at the nonpermissive temperature, failed to accumulate H1 capsid protein or progeny SS DNA but RF DNA replication still occurred.

The failure to accumulate AAV proteins is unlikely to be a result of SS DNA inhibition. For the ts125 mutant we showed elsewhere that there is a relatively normal level of AAV RNA transcription but a failure to accumulate certain spliced AAV mRNAs (10). An alternate hypothesis which seems less likely is that L-canavanine or the ts125 mutation inhibits an adeno-
superinfected with adenovirus (5 plaque-forming units/cell), at 37°C were infected with wild type intracellular viral DNA. Cells infected with adenovirus alone in absence of L-canavanine (5 mM) as described in the text. Intracellular viral proteins or capsids might be required either directly or merely to sequester SS DNA in particles and thus avoid recycling through the replicating pool. Neither of these hypotheses are mutually exclusive and further studies are required to distinguish between them. However, our current studies are consistent with the idea that free SS DNA exists only when sequestered in a capsid.

In monkey cells, adenovirus growth is restricted because of a failure to express efficiently certain late functions (20–22). Buller et al. (23) recently reported that AAV grown in monkey cells with only adenovirus as a helper synthesized efficiently AAV RF DNA but failed to accumulate all three AAV proteins. These same authors (23) cited unpublished experiments indicating that AAV SS DNA also failed to accumulate. These observations appear to be analogous to our results with the ts125 mutant in permissive human cells (10). In monkey cells, adenovirus DNA replication occurs normally. This indicates that the 72,000 protein, which is required stoichiometrically, is made in normal amounts. Thus, the restriction of AAV SS DNA with the ts125 mutant is more likely due to the absence of AAV proteins (as noted above) than to a direct requirement

\[ \text{AAV Capsid and SS DNA Accumulation} \]

\[ \text{TABLE I} \]

\textbf{Effect of L-canavanine on synthesis of AAV particles}

Cells were infected with wild type adenovirus and AAV, treated with L-canavanine (5 mM), and labeled with \(^{3}H\)thymidine and \(^{35}S\)methionine as described in the text and Fig. 1. AAV particles (full or empty) were quantitated as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Radioactivity incorporated</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full particles</td>
<td>104,509</td>
</tr>
<tr>
<td>Empty particles</td>
<td>68,470</td>
</tr>
<tr>
<td>+ L-canavanine</td>
<td>2,184</td>
</tr>
<tr>
<td>- L-canavanine</td>
<td>3,294</td>
</tr>
</tbody>
</table>

\[ \text{TABLE II} \]

\textbf{Effect of mutant adenovirus helper on synthesis of AAV particles at 40°C}

Cells were infected with AAV and either wild type adenovirus or adenovirus ts125 as helper, grown at 40°C, and labeled with \(^{3}H\)thymidine or \(^{35}S\)methionine as described in the text and Fig. 2. AAV particles (full or empty) were quantitated as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Wild type adenovirus</th>
<th>Adenovirus ts125 helper</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV full particles</td>
<td>918,000</td>
<td>21,000</td>
</tr>
<tr>
<td>AAV empty particles</td>
<td>538,000</td>
<td>13,000</td>
</tr>
</tbody>
</table>

\[ \text{Fig. 1. Effect of L-canavanine on intracellular AAV DNA.} \]

Cells grown at 37°C were infected with wild type adenovirus (5 plaque-forming units/cell), superinfected with AAV inoculum (2 infectious units/cell), and treated with L-canavanine (5 mM) as described in the text. Intracellular viral DNA was sedimented in neutral sucrose gradients. A, cells infected with adenovirus + AAV in the absence (O—O) or presence (●—●) of L-canavanine. B, cells infected with adenovirus alone in absence (O—O) or presence (●—●) of L-canavanine. (Δ—Δ), mock-infected cells.

\[ \text{Fig. 2. Effect of a temperature-sensitive mutation in the helper virus on intracellular AAV DNA.} \]

Cells were simultaneously infected with purified AAV (10 infectious units/cell) and adenovirus (10 plaque-forming units/cell) and grown at 40°C as described in the text. Intracellular DNA was sedimented in neutral sucrose gradients. A, cells infected with wild type adenovirus 5 + AAV (O—O) or Ad5 ts6 + AAV (●—●); B, cells infected with wild type adenovirus 5 (O—O) or Ad5 ts125 (●—●) alone.
for the 72,000 protein in AAV DNA replication. On the other hand, the adenovirus host range restriction in monkey cells is controlled apparently by other functions of this same E 72,000 protein (24). Recent studies suggest that one of these functions appears to be an effect on splicing of certain adenovirus (25) and AAV (10) mRNAs. Thus, the results of our studies of AAV and adenovirus in permissive human cells appear to be consistent with those of others in restrictive monkey cells.

The experiments reported here provide two independent ways to dissociate AAV SS DNA accumulation from RF replication. These procedures should provide possible approaches to analyze the mechanism of AAV SS DNA production. Also together with previous studies (10), this work provides an alternative approach to studying certain properties of the apparently multifunctional adenovirus E 72,000 protein in permissive human cells.

REFERENCES