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NOTES

Helper-dependent Infectious Deoxyribonucleic Acid from Adenovirus-associated Virus

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The defectiveness of adenovirus-associated viruses (AAV) is now well known (1, 8, 9, 11). Adenoviruses from widely divergent phylogenetic hosts, including man and monkeys (1), dogs (8), mice (M. D. Hoggan and N. R. Blacklow, *unpublished data*), and chickens (4) can serve as helpers for the formation of AAV in cell cultures. Furthermore, in the human host AAV replication is apparently dependent upon adenovirus infection (2).

Although it has been shown that the helper effect of adenovirus occurs late in the adenovirus replicative cycle (3, 9), the step in the AAV replicative cycle which is blocked in the absence of adenovirus helper is not known. To examine directly whether AAV replication is defective at a stage before or after the intracellular release of its genome (uncoating), we have tested purified AAV deoxyribonucleic acid (DNA) for infectivity. AAV-1 DNA is double-stranded (10) and similar in size to DNA isolated from polyoma virus and simian virus 40 (5, 12). The DNA of these papovaviruses has been shown to be infectious (6, 7). In the present study, AAV DNA was also found to be infectious, but only in cells coinfected with adenovirus.

AAV-1 (strain H) was grown with the AAV-free E46$^-$ strain of adenovirus type 7(E46$^-$) as helper (8) in human embryonic kidney (HEK) cells obtained from the Viral Carcinogenesis Branch of the National Cancer Institute. AV-1 A was also produced in a continuous line of rhesus monkey kidney cells (MK-2) infected with simian virus 15 (SV-15), both kindly provided by Dr. Atchison. AAV-1 was purified from infected cells by genetron extraction and isopycnic sedimentation in CsCl, and its DNA was extracted by treatment with papain, trypsin, sodium dodecylsulfate, and phenol (10). DNA samples were divided into equal portions (experiment 1, 15 μg; experiment 2, 24 μg). The indicated samples (Table 1) were incubated for 1 hr at 37 C with 300 μg/ml of electrophoretically purified deoxyribonuclease I (Worthington Biochemical Corp., Freehold, N.J.) in 0.15 ml of 0.01 M tris(hydroxymethyl)aminomethane buffer, *p*H 7.0, and 0.02 M $MgCl_2$. After digestion, enzyme-treated and untreated samples were adjusted to 0.2 ml in 0.55 M NaCl and tested in HEK cells for infectivity with and without E46$^-$ virus as helper.

Secondary monolayer cultures of HEK cells were prepared on cover slips in 50-mm petri dishes (Falcon Plastics, Los Angeles, Calif.). When confluent (approximately 10^6 cells), they were infected with E46$^-$ adenovirus at an input multiplicity of 3 to 6 $TCID_{50}$/cell or were mock-infected with tissue culture medium. Four hours later, all cultures were osmotically shocked by rapid, successive exposure to 5 ml of 0.37 M NaCl and 0.55 M NaCl. (This procedure did not affect the yield of virus in cells infected with AAV.) The various preparations indicated in Table 1 were then added to the cultures. After adsorption for 25 min at 29 C, the cultures received 5 ml of Eagle's medium containing 20% fetal calf serum and were placed in 5% CO_2 at 37 C. After a single cycle of virus replication (24 hr), the cover slips were removed and strained for AAV antigen by the indirect immunofluorescence technique (3). At this time, early adenovirus cytopathic changes were evident. The cells remaining on the dishes (approximately 5×10^5 cells) were incubated an additional 3 to 4 days until all cells were destroyed. The tissue culture fluids from these cultures were then tested for AAV-1 complement-fixing (CF) antigen (8). In addition, fresh HEK cultures were inoculated with 0.2 ml of a 1:30 dilution of the remaining culture fluids, and the harvesting procedure was repeated. To insure that the cells used in the experiments reported here were not contaminated with AAV, fluids from control cultures were passed repeatedly with E46$^-$ adenovirus as helper; no AAV was found.

As shown in Table 1, AAV was detected in cells

TABLE 1. *Detection of AAV-1 immunofluorescent (FA) and CF antigens*

Inoculum	E46⁻ adenovirus	Primary infection		1st passage of progeny	
		FA-positive cells	CF antigen titer	FA-positive cells	CF antigen titer
		%		%	
Expt 1[a]					
AAV-1 DNA	+	0.01	1	0.1	2
AAV-1 DNA + deoxyribonuclease	+	0	0	0	0
AAV-1 virus[b]	+	1.0	>4	0.45	2
Culture medium	+	0	0	0	0
AAV-1 DNA	−	0	0	0	0
Culture medium	−	0	0	0	0
Expt 2[c]					
AAV-1 DNA	+	0.03	8	4.4	64
AAV-1 DNA + deoxyribonuclease	+	0	0	0	0
AAV-1 virus[b]	+	4.6	64	2.9	32
Culture medium	+	0	0	0	0
AAV-1 DNA	−	0	0	0	0
Culture medium	−	0	0	0	0

[a] AAV-1 DNA extracted from AAV-1 grown in HEK cells with adenovirus 7 and used at a concentration of 15 μg per test plate.

[b] AAV-1 virus used at 3 $TCID_{50}$ per cell.

[c] AAV-1 DNA extracted from AAV-1 grown in MK-2 cells with SV15 and used at a concentration of 24 μg per test plate.

incubated with AAV DNA when adenovirus helper was present. Progeny particles from cells infected with AAV virions or the viral DNA were morphologically identical when examined by electron microscopy and antigenically indistinguishable by immunofluorescence and CF testing. Digestion of the AAV DNA with deoxyribonuclease completely eliminated its infectivity. AAV virions remain infectious after incubation with deoxyribonuclease under the same conditions.

In these experiments, the processes of AAV adsorption, penetration, and uncoating were circumvented by the use of viral DNA. However, as in cells infected with whole virus, adenovirus was still required for AAV synthesis. In addition to demonstrating that biologically active nucleic acid can be isolated from purified AAV, the experiments indicate that the defective step(s) in AAV replication occurs at some stage after the intracellular release of its DNA.

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