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Adenovirus-Associated Virus Multiplication

VII. Helper Requirement for Viral Deoxyribonucleic Acid and Ribonucleic Acid Synthesis

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The adsorption of adenovirus-associated virus (AAV) type 2 by KB cells and the subsequent penetration of the AAV genome to the cell nucleus was measured with and without helper adenovirus type 2 (Ad2). It was found that the helper virus did not enhance either process. On the other hand, a synthesis of AAV deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) was not detected by nucleic acid hybridization after KB cells were infected with AAV type 2 alone, whereas both AAV DNA and RNA synthesis were readily detected when cells were additionally infected with Ad2 or herpes simplex virus type 1, a partial helper of AAV replication. AAV RNA synthesis was initially observed 10 to 11 hr after simultaneous infection with Ad2, but the interval between AAV infection and AAV transcription could be reduced to 4 to 5 hr when cells were first infected with Ad2 for 10 hr. It was estimated that AAV DNA synthesis accounted for 3% of the total DNA in cells after a simultaneous infection with Ad2. These findings, together with the previous observation that adenovirus provides a helper function(s) after AAV uncoating, suggest that AAV are defective only subsequent to the uncoating process, and that helper viruses may provide a factor(s) needed for initiating synthesis of AAV DNA, RNA, or both.

Since it was first observed that adenoviruses served as helpers for the multiplication of adenovirus-associated viruses (AAV; 2), other viruses capable of inducing AAV multiplication have not been found. However, it has been demonstrated that herpesviruses can evoke AAV antigen synthesis without a detectable increase in AAV infectious titer or electron microscopic evidence of virion production (1, 4). Thus, herpesviruses appear to act as partial helpers of AAV replication. At present, only adenoviruses and herpesviruses are known to provide a function(s) required for AAV synthesis (1, 2, 4).

The molecular basis for the helper activity of adenoviruses and herpesviruses is as yet undefined. Except for their ability to assist AAV replication, these species are distinctive. Genetic relatedness between either class of helpers or between helpers and AAV has not been demonstrable (17; J. A. Rose, *unpublished data*). Furthermore, the AAV differ considerably from their helpers with respect to virion and genomic structure (3, 16, 22). In the present study, we have attempted to gain insight into the mechanism of AAV dependence by investigating the influence of helper virus on (i) the uptake of AAV by cells and (ii) the subsequent replication and transcription of the AAV genome. It was found that helper adenovirus type 2 (Ad2) did not exert an enhancing effect on the cellular adsorption or penetration of AAV type 2 (AAV-2). However, AAV-2 deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis could be detected only whe cellsn were also infected with Ad2 or a helper herpesvirus [herpes simplex virus type 1 (HSV-1)]. Based on the onset of AAV transcription in cells infected with helper Ad2, a factor(s) required for AAV RNA synthesis was apparently produced between 6 and 10 hr after adenovirus infection.

MATERIALS AND METHODS

Materials. The sources of KB cells, enzymes, thymidine-*methyl*-^3H (18 Ci/mmole), tritiated ribonucleoside 5'-triphosphates (3 to 4 Ci/mmole), and ^{32}P have been given (3, 16). Uridine-5-^3H (27 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass. HEp-2 cells were obtained from the National Institutes of Health Media Unit.

Viruses. Ad2 and AAV-2 were grown in KB cells in suspension culture and assayed as described previously (3). Procedures for purifying both viruses have also been described (19). HSV-1 (oral strain), originat-

ing from National Institutes of Health isolate 1828, was obtained from A. Rabson. A virus stock, prepared by passage in primary African green monkey kidney cells, had an infectivity titer of 10^7 plaque-forming units (PFU)/ml when plaqued on Vero cells (13). For preparative yields, HSV-1 was grown in HEp-2 cells according to a method reported by Roizman and Spear (15). Virus was released from cells by sonic treatment and was purified by a combination of nuclease treatment and differential centrifugation (20).

Measurements of viral adsorption and penetration. AAV-2 containing DNA labeled with ^{3}H-thymidine and Ad2 containing DNA labeled with ^{14}C-thymidine were produced as before (3, 16); the purified virus stocks had infectivity titers of 10^{10} and 10^{11} tissue culture infective dose ($TCID_{50}$) units/ml, respectively. Prior to infection, the ^{3}H-AAV inoculum was heated for 10 min at 56 C to inactivate any contaminating adenovirus (9). To measure the binding of radioactive virus to KB cells at specified times after infection, cells from 10-ml samples of spinner culture were collected, washed once with medium, solubilized in 1 ml of 0.1 N NaOH by heating for 1 hr at 80 C, and counted in a Triton X-100 emulsion (16). Significant additional counts were not recovered from cell samples by further washing prior to solubilization. Viral radioactivity associated with cells was resolved into nuclear and cytoplasmic fractions exactly as described by Penman (12). Cells were infected for 2 hr, washed once, resuspended in fresh medium, and incubated for an additional 18 hr. Cells from 100-ml samples were analyzed at indicated times after infection. Radioactivity in cytoplasmic fractions was determined from 0.5-ml samples which were mixed with an equal volume of 1 N NaOH, heated, and counted as above. To assay nuclear fraction radioactivity, nuclear pellets were solubilized in 2 ml of 0.5 N NaOH, and 0.2-ml samples were counted. ^{3}H and ^{14}C counts in each sample were corrected for channel spill.

Purification of DNA. The extraction of DNA from preparations of purified Ad2 and AAV-2 has been described (16, 17). DNA was extracted from HSV-1 by incubation with Pronase (1 mg/ml) in the presence of 0.3% sodium dodecyl sulfate (SDS) for 12 hr at 37 C. After phenol extraction, DNA was dialyzed into SSC (0.15 M NaCl, 0.015 M sodium citrate). For the recovery of DNA from infected and uninfected KB cells, cells from 200-ml samples of spinner culture were suspended in 4 ml of saline-EDTA (0.15 M NaCl, 0.1 M ethylenedinitrilo-tetraacetic acid, *p*H 8.0), SDS was added to a final concentration of 0.3%, and the mixture was incubated overnight at 37 C with Pronase (200 μg/ml). An addition of 0.4 ml of 1 M tris(hydroxymethyl)aminomethane (Tris), *p*H 8.6, was then made, and the mixture was incubated with trypsin (100 μg/ml) for 2 hr at 37 C. After the SDS concentration had been increased to 3%, the mixture was given a final incubation at 50 C for 30 min, extracted three times with phenol, and dialyzed into SSC. The DNA content of samples was estimated by absorbance at 260 nm after hydrolysis of RNA with 0.2 N KOH and removal of ribonucleotides by dialysis into SSC. Approximately 25 to 30% of initial absorbance remained after this treatment. When AAV virions containing ^{3}H-thymidine-labeled DNA were added to cells, 60 to 70% of the radioactivity was recovered in DNA extracts.

Preparation of RNA. RNA synthesized in 200-ml samples of infected and uninfected cells was labeled with ^{3}H-uridine (1 μCi/ml) or ^{32}P (4 μCi/ml) during periods specified in individual experiments. For ^{32}P labeling, cells were resuspended in phosphate-free medium containing 5% dialyzed horse serum. Pulses were terminated by collecting cells in centrifuge bottles which were chilled to −60 C. Both ^{3}H- and ^{32}P-RNA were purified as described previously (19), except that ^{3}H-RNA preparations were not chromatographed on Sephadex G-25 but instead were incubated with Pronase (200 μg/ml) for 1 hr (after deoxyribonuclease treatment) and again extracted with hot phenol. In vitro-synthesized AAV-2, Ad2 and HSV-1 ^{3}H-RNA were prepared as before (19).

Nucleic acid hybridization. DNA-RNA hybrids were formed on nitrocellulose filters and detected in a liquid scintillation spectrometer (19). In all instances, hybridization results represent averages of duplicate determinations. Blank filter values (0.005% or less of added in vivo RNA radioactivity and 0.01% or less of added synthetic RNA radioactivity) have been subtracted. Parallel hybridization experiments with radioactive AAV-2 and Ad2 DNA indicated that essentially all DNA initially retained by filters remained bound at the end of the hybridization procedure.

RESULTS

AAV adsorption and penetration. It was shown previously that AAV DNA is infectious, but only when cells were also infected with an adenovirus (10). Although this finding indicated that AAV replication was defective at a step(s) subsequent to AAV uncoating, there remained a possibility that helper adenovirus also exerted a quantitative effect on AAV adsorption and penetration. The adsorption of purified AAV-2 by KB cells infected with and without Ad2 is shown in Fig. 1. AAV was labeled with ^{3}H-thymidine and banded three times in CsCl density gradients. When centrifuged through a 5 to 20% sucrose gradient, the virus sedimented as a single homogenous component with an $S_{20,w}$ of 120 relative to polyoma virus [$S_{20,w}$ = 238 (7)]. The data in Fig. 1 show that AAV adsorption was about the same with or without helper adenovirus when the effect of adenovirus was tested at multiplicities of 1, 10, and 100 $TCID_{50}$ units/cell. Adsorption was rapid (75% complete in 30 min), and the maximal fraction of bound virus was observed by 1 to 2 hr after infection. In KB cells similarly infected with AAV (10 $TCID_{50}$ units/cell), AAV multiplication is efficiently supported by adenovirus multiplicities of 10 to 50 $TCID_{50}$ units/cell (see below). Because further increases in the multiplicity of

AAV infection may reduce the yield of infectious AAV (J. A. Rose et al., *unpublished data*), an AAV multiplicity of 5 to 10 $TCID_{50}$ units/cell was used in experiments described in this paper. For the experiment shown (Fig. 1), a total of 25 to 30% of added AAV was adsorbed. Assuming random labeling and adsorption of particles, this was equivalent to the binding of about 3 $TCID_{50}$ units/cell. In other experiments, total AAV binding was 30 to 35% and 15 to 19% (Table 1), and the presence of adenovirus clearly did not alter AAV adsorption.

AAV DNA radioactivity was also measured in nuclear and cytoplasmic fractions of KB cells at 2, 10, and 20 hr after single or dual infection to determine whether penetration to the cell nucleus was influenced by adenovirus (Table 1). Over this entire period, nuclear preparations contained 30 to 45% of cell-associated AAV counts. In control experiments, only 3% of virus counts were recovered with cell nuclei when virus (^{3}H-AAV or ^{14}C-adenovirus) was added to disrupted cells. At each time point, the percentage of nuclear AAV counts was nearly the same with or without adenovirus infection and was equivalent to about 1 $TCID_{50}$ unit/cell. In addition, there was an increase in the proportion of nuclear counts from 30 to 35% at 2 hr to 44 to 45% at 20 hr after infection. The association of helper adenovirus DNA radioactivity with cell nuclei is also shown in Table 1. The percentage of adenovirus counts found in nuclear fractions was roughly comparable to that seen with AAV and was equivalent to about 4 $TCID_{50}$ units/cell.

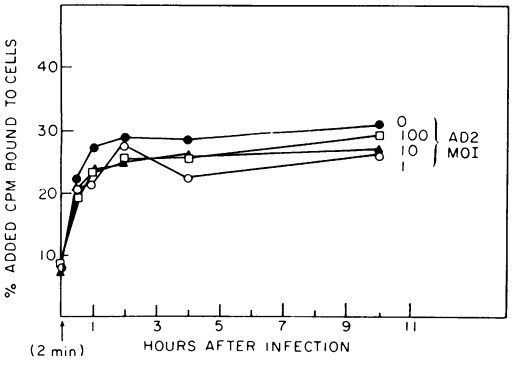

FIG. 1. *Adsorption of AAV by KB cells with and without helper adenovirus. A 400-ml spinner (2.8 × 10⁵ cells/ml) was infected with ^{3}H-AAV-2 (4.2 × 10⁵ counts/min) at a multiplicity of 10 $TCID_{50}$ units/cell and divided into four cultures which were additionally infected with the indicated multiplicities (MOI) of adenovirus.*

It is concluded from the above experiments that helper adenovirus affects neither the adsorption of AAV nor the subsequent penetration of the AAV genome to the cell nucleus. Because of the apparent lack of adenovirus influence on AAV adsorption, penetration, and uncoating, AAV dependence on adenovirus was studied at the levels of transcription and DNA synthesis.

AAV transcription. To determine whether transcription of the AAV genome required adenovirus co-infection, RNA from cells infected with Ad2, AAV-2, or both was tested for its ability to hybridize with AAV DNA (Table 2). RNA was labeled with ^{3}H-uridine for 3-hr intervals after infection. Cells infected with adenovirus alone contained RNA that hybridized with adenovirus DNA but not with AAV DNA (Table 2, I). This finding suggests that AAV DNA does not hybridize with any of the total adenovirus RNA transcript, because all species of adenovirus RNA should be labeled between 17 and 20 hr (8). When cells were infected with

TABLE 1. *AAV DNA radioactivity in the cytoplasmic and nuclear fractions of KB cells infected with and without helper adenovirus*

Virus	Time after infection (hr)[a]	Cytoplasmic fraction counts/min[b]		Nuclear fraction counts/min[b]		Added counts/min bound to cells (%)		Bound counts/min in nuclear fraction (%)	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
I. ^{3}H-AAV-2	2	14,584	—	7,984	—	16	—	35	—
	10	13,494	—	7,268	—	15	—	36	—
	20	12,663	—	9,799	—	16	—	44	—
II. ^{3}H-AAV-2 + ^{14}C-Ad2	2	18,181	19,046	7,766	19,242	18	14	30	50
	10	19,172	19,838	11,230	15,190	22	12	37	45
	20	14,429	15,680	12,020	18,105	19	12	45	54

[a] Two 300-ml spinners (3.6 × 10⁵ cells/ml) were each infected with purified ^{3}H-AAV-2 (4.2 × 10⁵ counts/min) at a multiplicity of 10 $TCID_{50}$ units/cell, and one spinner (II) was also infected with purified ^{14}C-Ad2 (2.8 × 10⁵ counts/min) at a multiplicity of 55 $TCID_{50}$ units/cell.

[b] Counts/min recovered from cells in 100-ml samples of spinner culture. Averages of duplicate determinations are shown.

TABLE 2. *Adenovirus requirement for AAV RNA synthesis*

Virus[a]	Pulse interval after infection[b]	^{3}H-RNA counts/min incubated with DNA	^{3}H-RNA counts/min bound to		AAV-2 titer[d]
			AAV-2 DNA[c]	Ad2 DNA[c]	
I. Ad2	11–14	366,696	18	1,751	—
	17–20	344,305	14	3,316	0
II. AAV-2[e]	−3–0	485,682	3	—	—
	3–6	573,328	3	—	$10^{4.0}$
	7–10	427,097	3	—	—
	17–20	405,450	0	—	$10^{4.0}$
(+ Ad2)	17–20	71,801	447	—	$10^{7.0}$
III. AAV-2[e] + Ad2	−3–0	508,116	0	0	$10^{4.0}$
	3–6	490,487	24	58	$10^{4.0}$
	7–10	400,162	13	199	$10^{4.0}$
	11–14	559,134	122	1,974	$10^{4.0}$
	17–20	281,785	847	2,369	$10^{7.0}$

[a] A 500-ml (I) and two 1,000-ml (II and III) KB spinners (2.7×10^5 cells/ml) were infected with AAV and adenovirus as indicated at multiplicities of 5 and 10 $TCID_{50}$ units/cell, respectively.

[b] Two samples (−3–0) served as preinfection controls.

[c] Filters contained 2 μg of viral DNA. Only binding which exceeds twice the blank filter value has been taken to be significant.

[d] $TCID_{50}$ units per milliliter of cells at end of pulse interval.

[e] The AAV inoculum was heated at 56 C for 10 min.

AAV alone, neither AAV nor adenovirus RNA was found, and AAV infectivity did not increase (Table 2, II). The AAV inoculum used in this experiment had been heated to inactivate contaminating adenovirus. To be certain that heating did not also inactivate AAV, a portion of the AAV-infected cells was simultaneously infected with adenovirus. At 20 hr, the AAV infectivity of these cells was increased 1,000-fold, and they contained RNA that hybridized with AAV DNA. The detection of AAV and adenovirus RNA at intervals after simultaneous infections is shown in the next experiment (Table 2, III). Adenovirus transcription was detected at 3 to 6 hr, whereas AAV transcription was first seen at 11 to 14 hr. AAV infectivity was again elevated in the 20-hr sample. These experiments indicate that (i) the AAV genome is not transcribed unless cells are also infected with adenovirus and (ii) after dual infection adenovirus transcription precedes the onset of AAV transcription by at least 5 hr. A more detailed analysis of AAV transcription is given in Fig. 2. Figure 2A shows the time course of viral RNA synthesis after a simultaneous infection with AAV and adenovirus. In this experiment, RNA labeled with ^{32}P for 1-hr intervals was reacted with AAV and adenovirus DNA. AAV RNA was possibly present at 9 to 10 hr and was clearly evident 10 to 11 hr after infection. Again, adenovirus RNA was found at least 5 hr before AAV RNA was detected. After onset, the rate of AAV RNA synthesis increased exponentially.

The period between AAV infection and the appearance of AAV RNA could be shortened if cells were first infected with adenovirus for 10 hr. The time course of AAV RNA synthesis under these conditions is shown in Fig. 2B. RNA was labeled with ^{3}H-uridine for 1-hr intervals and then hybridized with AAV DNA. AAV RNA was definitely detected 4 to 5 hr after AAV infection. The relative increases in AAV RNA synthesis were similar to those observed after simultaneous infection (Fig. 2A). Also shown is a control experiment in which labeled RNA from cells infected with only adenovirus was incubated with both AAV and adenovirus DNA. This RNA did not hybridize with AAV DNA, again indicating the specificity of AAV RNA detection.

AAV DNA synthesis. In vitro-synthesized, virus-specific RNA was used to detect and quantitate AAV and adenovirus DNA (14, 17) in DNA extracted from infected cells. A proportionality exists between the fraction of added synthetic RNA hybridized and the amount of homologous viral DNA present in mixtures with cell DNA (Fig. 3). Hybridization reactions between the same amounts of these synthetic RNA preparations and DNA extracted from cells at different times after a simultaneous infection are shown in Table 3. A synthesis of both AAV-2 and Ad2 DNA is indicated by the increased binding of AAV-2 and Ad2 synthetic RNA to DNA extracted at 15 hr or later. No increases in AAV synthetic RNA binding were observed when hybridizations were carried out with DNA extracted from cells infected only with AAV-2 or

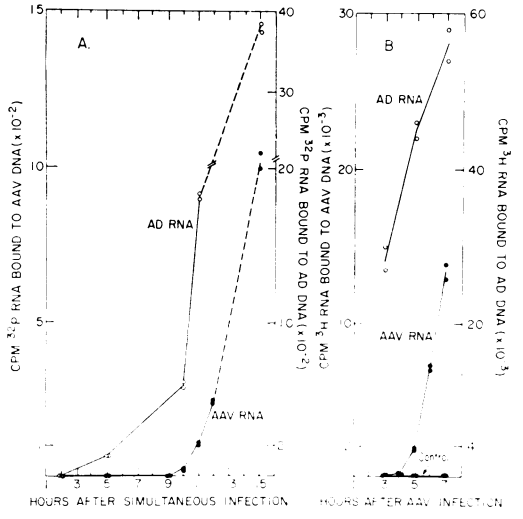

FIG. 2. *Time course of AAV and adenovirus RNA synthesis. KB cell suspension cultures (3.0 × 10⁵ cells/ml) were infected with multiplicities of 10 and 50 $TCID_{50}$ units/cell of AAV-2 and Ad2, respectively. (A) A 1,500-ml spinner was simultaneously infected and RNA was labeled with ^{32}P for 1-hr periods after infection (except for the 15-hr sample, which was labeled for 3 hr). Extracted ^{32}P-RNA was hybridized with nonlimiting quantities of viral DNA (4 μg), and counts per minute in each preparation which bound to AAV-2 DNA (AAV RNA) and Ad2 DNA (AD RNA) are plotted. Binding has been normalized to ^{32}P-RNA inputs of 1.5 × 10⁵ counts/min (^{32}P-RNA additions ranged from 1.0 × 10⁵ to 1.5 × 10⁵ counts/min). Extrapolations from 12 hr (1-hr pulse) to 15 hr (3-hr pulse) values are indicated by dashed lines. (B) A 1,800-ml spinner was infected with Ad2 for 10 hr; 1,000 ml was then split off and infected with AAV-2. Samples from both spinners were labeled with ^{3}H-uridine for 1-hr periods subsequent to the time of AAV infection. ^{3}H-RNA extracted from AAV-infected samples was hybridized with AAV-2 DNA, and counts per minute bound (AAV RNA) were normalized to inputs of 3.0 × 10⁶ counts/min. ^{3}H-RNA extracted from samples infected with only Ad2 was hybridized with both Ad2 and AAV-2 DNA, and counts per minute bound (AD RNA and control, respectively) were similarly normalized. All ^{3}H-RNA additions ranged from 2.0 × 10⁶ to 3.0 × 10⁶ counts/min, and reactions were carried out with nonlimiting amounts of viral DNA (4 μg).*

Ad2 In other hybridization experiments with pulse-labeled DNA, AAV DNA synthesis has not been detected in the absence of helper Ad2 (J. A. Rose et al., *in preparation*). With synthetic RNA, low levels of binding also occurred with early DNA preparations. These reactions may have been due to absorbed virus or the collection with cell samples of viral DNA (associated with cell fragments) added in the crude inocula, or to both.

Reactions with synthetic AAV and adenovirus RNA appear to plateau by 30 to 40 hr (Table 3). RNA was not limiting in these reactions, since larger fractions of each RNA addition could be hybridized (Fig. 3). Thus, based on calibration plots shown in Fig. 3, the plateau values of RNA binding can be related to the quantity of AAV-2 and Ad2 DNA in late samples of extracted DNA. These estimates indicate that the net synthesis of AAV and adenovirus DNA was about equal, approximately 148 ng of each DNA per 5 μg of extracted DNA, if it is assumed that the efficiency of recovery of each viral DNA was similar. If it is further assumed that cell and viral DNA were recovered with the same efficiency, each viral DNA would account for 3% of the total DNA. For AAV, this is equivalent to 2 × 10⁵ to 4 × 10⁵ genomes per cell, a number 10 to 100 times greater than that calculated to account for cellular infectivity (Table 3) when an infectious unit is taken to represent a single virus genome. The above number of genomes per cell was calculated as follows: based on an estimated KB cell DNA content of 2 × 10^{-11} to 4 × 10^{-11} g (22), the AAV DNA content per cell would be 3% of 2 × 10^{-11} to 4 × 10^{-11} g or 0.06 × 10^{-11} to 0.12 × 10^{-11} g, and the number of AAV genome equivalents per cell would be equal to 0.06 × 10^{-11} g to 0.12 × 10^{-11} g, divided by 1.8 × 10⁶ [the molecular weight of AAV genome (16)] and multiplied times 6.02 × 10^{23} (Avogadro's number).

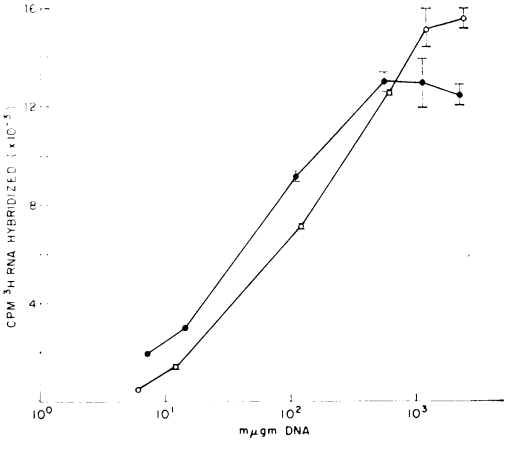

FIG. 3. *Detection and assay of AAV-2 and Ad2 DNA by hybridization with synthetic, virus-specific ^{3}H-RNA. Each viral DNA sample was mixed with 5 μg of KB cell DNA. Filters containing AAV DNA were reacted with additions of 24,675 counts/min of AAV-2 synthetic RNA, and filters containing adenovirus DNA were reacted with additions of 17,070 counts/min of Ad2 synthetic RNA. (●) AAV RNA binding; (○) adenovirus RNA binding.*

It can be seen (Table 3) that cellular AAV infectivity increased exponentially for 9 hr after the apparent cessation of viral DNA synthesis. At least 90% of the total yield of infectious AAV was produced during this interval, suggesting that AAV maturation does not require concomitant viral DNA synthesis. It was also observed that AAV infectivity was predominantly cell-associated during the course of infection (cells contained 1,000-fold more infectivity than the supernatant medium at 30 hr).

Induction of AAV RNA and DNA synthesis with herpes simplex virus. Because AAV antigen is synthesized in cells which are co-infected with herpesviruses (1, 4), AAV RNA synthesis should occur, and hybridization tests would be expected to detect this RNA. Both in vivo- and in vitro-synthesized AAV-2 and HSV-1 RNA are highly specific for their respective DNA templates [J. A. Rose, *unpublished data*; see accompanying paper (6)]. The detection of AAV RNA in KB cells simultaneously infected with heated AAV-2 and HSV-1 is shown in Table 4. AAV RNA was not seen 2.5 to 5.5 hr after infection but was present in the 7 to 10-hr sample and was considerably increased by 17 to 20 hr. The induction of AAV RNA synthesis was due to HSV-1 because other experiments demonstrate that this helper activity, in contrast to that of adenovirus, is abolished by chloroform extraction of the virus inoculum [see accompanying paper (6)]. As was the case with Ad2, HSV-1 transcription occurred before AAV RNA synthesis could be detected. RNA complementary to Ad2 DNA was not found, and there was no increase above the input AAV titer seen at 2.5 hr after infection. The relative amount of AAV RNA synthesized between 17 and 20 hr was comparable to that observed with adenovirus as a helper (Table 2). Table 4 further shows that, in addition to its helper activity for AAV RNA synthesis, HSV-1 also provides a function(s) required for AAV DNA synthesis. AAV and HSV-1 DNA synthesis was detected with synthetic RNA as described for the previous experiment (Fig. 3 and Table 3). There was no discernible synthesis of Ad2 DNA, evidence (along with the absence of Ad2 RNA) that the heated AAV stock contained no viable adenovirus which could serve as a helper. Based on a reconstruction experiment (Fig. 4) like that shown in Fig. 3, it is estimated that there

TABLE 3. *Synthesis of viral DNA and infectious AAV after a simultaneous infection of KB cells with AAV-2 and Ad2*

Time after infection (hr)[a]	Counts/min of added synthetic ^{3}H-RNA bound to extracted DNA[b]		AAV-2 titer ($TCID_{50}$ units/ml of cells)
	AAV RNA	Ad2 RNA	
3	33	410	$10^{6.0}$
6	31	402	$10^{6.5}$
9	43	495	$10^{5.5}$
12	60	453	$10^{5.5}$
15	1,050	874	$10^{6.0}$
18	5,497	2,917	$10^{6.5}$
21	7,719	4,524	$10^{7.5}$
30	7,771	5,826	$10^{10.0}$
40	7,479	5,924	$10^{9.0}$

[a] A 2,400-ml spinner (3.0×10^5 cells/ml) was infected with AAV-2 and Ad2 at multiplicities of 10 and 50 $TCID_{50}$ units/cell, respectively.

[b] The same amounts of each synthetic viral RNA preparation used in the preceding experiment (Fig. 3) were reacted with 5-μg samples of extracted DNA.

TABLE 4. *Synthesis of viral RNA and DNA and infectious AAV after a simultaneous infection of KB cells with AAV-2 and HSV-1*

Time after infection (hr)[a]	Counts/min of added in vivo ^{3}H-RNA bound to viral DNA[b]			Counts/min of added synthetic ^{3}H-RNA bound to extracted DNA[c]			AAV-2 titer ($TCID_{50}$ units/ml of cells)
	AAV DNA	HSV DNA	Ad2 DNA	AAV RNA	HSV RNA	Ad2 RNA	
2.5	—	—	—	553	664	81	$10^{5.0}$
5.5	0	436	—	546	717	79	$10^{4.0}$
10	212	2,218	0	2,105	1,996	64	$10^{4.0}$
20	2,214	3,509	0	21,188	7,160	47	$10^{5.0}$

[a] A 1,000-ml spinner (3.5×10^5 cells/ml) was infected with heated AAV-2 at a multiplicity of 10 $TCID_{50}$ units/cell and with HSV-1 at a multiplicity of 1 PFU/cell.

[b] RNA labeling periods were 2.5 to 5.5, 7 to 10, and 17 to 20 hr. ^{3}H-RNA (2.4×10^5 counts/min) was hybridized with 4 μg of each viral DNA. Approximately 0.02% of added ^{3}H-RNA (2×10^5 counts/min) from uninfected cells bound to 4 μg of HSV DNA.

[c] Each synthetic viral RNA was reacted with 10-μg samples of extracted DNA. Additions were: 69,840 counts/min, ^{3}H-AAV-2 RNA; 43,231 counts/min, ^{3}H-HSV-1 RNA; and 53,367 counts/min, ^{3}H-Ad2 RNA.

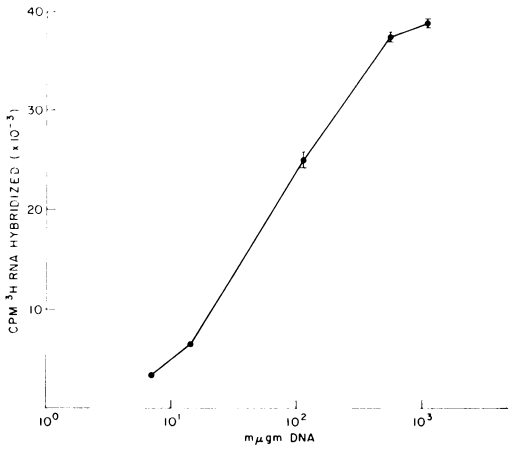

FIG. 4. *Detection and assay of AAV-2 DNA by hybridization with synthetic AAV-2 ^{3}H-RNA. Each AAV DNA sample was mixed with 10 μg of KB cell DNA. The amount of synthetic AAV-2 RNA incubated with each filter was the same as that used in the preceding experiment (Table 4).*

was 84 ng of AAV DNA per 5 μg of DNA extracted 20 hr after simultaneous infection. It is not certain whether this represents the maximal amount of AAV DNA which could have been synthesized, because samples beyond 20 hr were not assayed. However, the net amount of AAV DNA synthesized by 20 hr was 1.7% of the total DNA, or about half that observed when adenovirus was used as a helper (Fig. 3 and Table 3).

DISCUSSION

The lack of an adenovirus effect on AAV adsorption and penetration (Fig. 1 and Table 1), coupled with DNA infectivity data (10), suggests that adenoviruses assist AAV replication only after the intracellular release of AAV DNA (uncoating). An equivalent susceptibility of AAV DNA to deoxyribonuclease digestion after AAV infection with and without adenovirus has also been found (K. I. Berns, *personal communication*), additionally supporting this conclusion. On the other hand, AAV DNA and RNA synthesis could not be detected unless cells were also infected with either a helper adenovirus (Tables 2 and 3) or herpesvirus (Table 4).

With adenovirus as a helper, the interval between AAV infection and detectable AAV RNA synthesis was reduced from 10 to 11 hr to 4 to 5 hr, if cells were first infected with adenovirus for 10 hr (Fig. 2A and B). If the adenovirus requirement(s) for AAV transcription is no longer limiting 10 hr after adenovirus infection, 4 to 5 hr would represent the minimal delay between AAV infection and the onset of AAV RNA synthesis. Others have observed a similar delay between AAV infection and the detection of AAV antigen synthesis when cells were initially infected with adenovirus for 10 hr or longer (5, 11). Although our results suggest that a factor(s) promoting AAV transcription is made between 6 and 10 hr after adenovirus infection, an earlier presence of low, ineffective levels of this factor(s) cannot be ruled out.

After a simultaneous infection with adenovirus, the period during which most AAV DNA was synthesized was readily identified with synthetic AAV RNA (Table 3). Because AAV DNA and RNA synthesis has been detected only in infections with helper viruses, it should be considered that a helper-coded factor(s) might be directly responsible for initiating replication or transcription (or both) of the AAV genome. It is also possible, however, that some or all species of AAV DNA, RNA, or both are synthesized in the absence of a helper virus, but that the amounts made were too small to be detected by the techniques used.

The ability of HSV-1 to activate a synthesis of both AAV DNA and RNA (Table 4) and antigen (1, 4) indicates that its helper function is extensive. This conclusion is further supported by evidence that AAV-2 RNA synthesized with helper HSV-1 contains the full complement of AAV message [see accompanying paper (6)]. There may be other viruses which provide a more restricted support of AAV replication than is observed with HSV-1; i.e., they might only promote DNA and RNA synthesis. These viruses would not have been detected by tests for helper activity which depend on an induction of AAV antigen synthesis (1, 2).

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