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DNA-Minus Temperature-Sensitive Mutants of Adenovirus Type 5 Help Adenovirus-Associated Virus Replication

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Efficient potentiation of adenovirus-associated viruses (AAV) replication occurs in coinfections with either of two DNA-minus temperature-sensitive mutants of adenovirus type 5 (Ad5), ts125 and ts149. The helper activity of these mutants does not result from leakiness. At the nonpermissive temperature (39.5 C) there was little or no detectable adenovirus DNA synthesis, and only a relatively low level of adenovirus transcription was observed. However, the synthesis of AAV DNA and RNA and the yield of infectious AAV were comparable in amounts to those found when wild-type Ad5 was the helper. Furthermore, an apparent lag in the initiation of AAV transcription after the onset of AAV DNA synthesis was seen in coinfections with both wild type or ts125. These findings strongly suggest that the adenovirus factor(s) required for AAV multiplication is produced early in the adenovirus cycle. In addition, it is likely that if AAV multiplication is linked to adenovirus DNA replication, this requirement does not include all factors directly needed for adenovirus DNA synthesis.

Adenovirus-associated viruses (AAV) are defective, single-stranded DNA-containing parvoviruses (16). They are able to multiply only in cell cultures coinfected with a helper adenovirus. Attempts to determine which step(s) is defective in AAV replication have shown that AAV are capable of adsorption, penetration, and uncoating in the absence of helper, but that production of AAV DNA, RNA, and structural proteins requires coinfection with either an adenovirus (complete helper) or herpesvirus [(partial helper) 1, 3, 18]. Recently, a kinetic study of the temporal association between the appearance of adenovirus and AAV DNA and RNA has suggested that separate helper functions may be required for AAV DNA and RNA synthesis (5).

In the present study we have investigated these postulated levels of helper requirement using temperature-sensitive (ts) adenovirus mutants deficient in DNA synthesis at the nonpermissive temperature (39.5 C). Several such mutants have been isolated from adenovirus types 5, 12, and 31 and comprise two or possibly three complementation groups (7, 13, 14, 20, 21). ts125 and ts149 are adenovirus type 5 (Ad5) DNA-minus ts mutants from two distinct complementation groups (7). At 39.5 C these mutants reduce host DNA synthesis, synthesize T antigen, and transform cells, but they are restricted in the synthesis of their own DNA, and late viral RNA and proteins cannot be detected (7; T. H. Carter and H. S. Ginsberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S 250, p. 255; H. S. Ginsberg et al., unpublished data). Relative to the growth of the wild-type (WT) strain at 39.5 C, both mutants are restricted approximately 10^6-fold. In AAV coinfections with either mutant at 39.5 C, we have found that, despite the restriction of mutant DNA synthesis, AAV DNA, RNA, and infectious virions are synthesized in amounts comparable to those made in the presence of WT virus.

MATERIALS AND METHODS

Cells and viruses. KB cells were from a line orginally obtained from M. Green, and primary human embryonic kidney cells were purchased from Flow Laboratories Inc., Rockville, Md. It was shown previously that the WT strain of Ad5 grows well in KB suspension cultures within the temperature range of 32 to 39.5 C, and that the one-step growth curve at 39.5 C is accelerated approximately twofold relative to the growth at 32 C (7). Therefore, WT strain stocks were prepared at 37 C and harvested at 36 to 40 h postinfection, whereas stocks of ts125 and ts149 were prepared at 32 C and harvested at 65 h after infection. The infectivity of all stocks was determined at 32 and

39.5 C in human embryonic kidney monolayer cultures and expressed as mean tissue culture infective dose units. The results given in Table 1 confirm the marked restriction of growth of the ts strains at 39.5 C, i.e, approximately 1 infectious unit in a million is capable of replication at the nonpermissive temperature. Comparable results were obtained when infectivity was determined with a fluorescent focus assay (7).

AAV-2 stocks consisted of virus purified by banding three times in CsCl; contaminating adenovirus was inactivated by heating at 56 C for 10 min (2, 19). AAV infectivity was assayed in human embryonic kidney monolayer cultures as described previously (10).

Preparation of DNA and RNA. Purified, unlabeled AAV-2 and Ad5 DNA and ^{32}P-labeled Ad5 DNA were prepared by methods used previously. Marker ^{32}P-labeled simian virus 40 DNA was a gift of M. Thoren. The labeling of DNA or RNA in infected or uninfected cultures was carried out by pulsing with [^{3}H]thymidine (50 Ci/mmol) or [^{3}H]uridine (50 Ci/mmol) (both from Schwarz-Mann) at 10 μCi/ml for 1-h periods unless otherwise specified. Total DNA or RNA was recovered from infected or uninfected KB cells as before (18, 19), and the nucleic acid content of samples was estimated by absorbance at 260 nm. Viral DNA was also selectively extracted by a modification of the Hirt method (M. J. Ensinger, personal communication; 9). After labeling with [^{3}H]thymidine, a total of 6×10^6 infected cells were pelleted at $600 \times g$ and resuspended in 3 ml of 0.01 M Tris, 0.01 M EDTA, and 1% sodium dodecyl sulfate, pH 8. The sample was incubated at 37 C for 30 min, self-digested Pronase (30 min at 60 C, then 2 h at 37 C) was added to a final concentration of 500 μg/ml, and incubation was continued for another 2 h at 37 C. One milliliter of 4 M NaCl was added with gentle mixing at 0 C, and the sample was dialyzed overnight at 4 C against 0.01 M Tris, 0.01 M EDTA, and 1.0 M NaCl, pH 8. The supernatant was collected after centifugation at 15,000 rpm for 30 min at 4 C in a SW50L rotor and dialyzed against 0.1× SSC (0.015 M NaCl plus 0.0015 M sodium citrate) and 0.01 M EDTA at 4 C. Hybridization of counts in the supernatant and pellet fractions demonstrated that approximately 80% of the total Ad5- or AAV-specific counts were in the supernatant fraction.

Nucleic acid hybridization. All DNA-DNA and DNA-RNA hybridization reactions were performed in duplicate on nitrocellulose filter membranes (18, 24). Ad5 and AAV DNA and RNA do not cross-hybridize (5, 17). In all instances estimations of relative amounts of viral nucleic acid synthesis in the various samples were based on virus-specific counts per minute hybridized per 4 μg of total added DNA or per 8 μg of total added RNA. Experiments demonstrated that saturating conditions prevailed when these inputs of labeled DNA or RNA were reacted with 4 μg of purified viral DNA bound to the filter. To be certain of saturating conditions, however, each reaction was carried out with filters containing 4 or 8 μg of viral DNA. In no instance was the binding of counts significantly greater at the 8 μg level. Nonspecific binding was determined from reactions with labeled DNA or RNA extracted from uninfected cultures. These values have been subtracted and did not exceed 0.1% of added counts for DNA-DNA hybridizations or 0.01% of added counts for DNA-RNA hybridizations.

TABLE 1. *Growth of Ad5 strains at 32 and 39.5 C*

Strain	Titer ($TCID_{50}$/ml)[a]		Ratio of titer (39.5/32 C)
	32 C	39.5 C	
WT	2×10^{11}	2×10^{11}	1.0
ts125	1×10^{11}	3×10^{5}	3×10^{-6}
ts149	1×10^{10}	3×10^{4}	3×10^{-6}

[a] $TCID_{50}$, Mean tissue culture infective dose units.

Velocity sedimentation in neutral sucrose gradients. Hirt supernatant material was analyzed by velocity sedimentation through 5 to 30% neutral sucrose gradients containing 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, and 0.1% Sarkosyl, pH 8.0, in an SW41 rotor at 10 C in the presence of appropriate ^{32}P-labeled Ad5 and simian virus 40 DNA markers. Gradients were fractionated by puncturing the bottoms of centrifuge tubes and displacing equal volumes repeatedly with mineral oil injected by a Cornwall syringe.

Assay of radioactivity. Membrane filters containing labeled DNA-DNA or DNA-RNA hybrids were counted in liquifluor-toluene, and aqueous samples containing labeled DNA were counted in a Triton-X emulsion.

RESULTS

Viral DNA Synthesis at 32 C. That adenoviruses help the complete replication of AAV at 37 C has been well documented (10, 16). Therefore, the capability of both WT and the two DNA-minus ts strains of Ad5 to support AAV DNA replication at 32 C was examined initially. Suspension cultures inoculated with AAV alone or infected with each of the Ad5 strains with and without AAV coinfection were grown at 32 C for 27 h. Prior to harvesting the cells, DNA was labeled for 1 h with [^{3}H]thymidine. DNA was extracted from these cultures and hybridized with unlabeled Ad5 or AAV DNA. The DNA-DNA hybridization data (Table 2) demonstrate that at 32 C (i) AAV does not replicate autonomously (i.e., it is itself not a ts mutant whose growth is restricted at 37 C), (ii) comparable amounts of adenovirus specific DNA were synthesized by the three Ad5 strains during the pulse interval, (iii) comparable amounts of AAV DNA were synthesized in coinfections with each of the Ad5 strains during the pulse interval, and (iv) there was no detectable cross-contamination of the Ad5 and AAV stocks. For approximate quantitative comparisons of DNA synthesis in this and other experiments, it has been assumed that the specific

TABLE 2. *Virus-specific DNA synthesis in infections at 32 C*

Helper adenovirus	Without AAV coinfection		With AAV coinfection	
	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]
None			0[b]	0[b]
WT	2,262	10	2,703	3,040
ts125	4,917	0	2,235	3,201
ts149	2,111	6	2,549	1,484

[a] Expressed as total counts per minute bound per 4 μg of input DNA.

[b] Cells infected with AAV only.

activity of viral DNA was the same in each sample within sets of compared samples.

Viral DNA synthesis at 39.5 C. KB cell suspension cultures at 39.5 C were infected with each of the three Ad5 strains at input multiplicities in logarithmic increments. In each case DNA was labeled with [^{3}H]thymidine for 10 h starting at 10 h after infection. This long labeling period was chosen so that kinetic differences which might exist in the synthesis of viral DNA among the strains could be minimized. Extracted DNA from each culture and from an uninfected control was hybridized with Ad5 DNA to determine the relative amounts of adenovirus DNA synthesized during the labeling period at each input multiplicity of infection (Fig. 1). These data indicate a marked restriction of viral DNA synthesis by the ts mutants at the nonpermissive temperature. A small amount of DNA synthesis by the mutants could be definitely detected only at the highest added multiplicity.

KB cell suspension cultures at 39.5 C were then infected with AAV alone or coinfected with AAV and each adenovirus strain at the five different input multiplicities used in the previous experiment. After similarly labeling and extracting DNA, DNA-DNA hybridization revealed that AAV DNA could be synthesized in coinfections with each of the Ad5 strains but not in cells infected with AAV alone. The relative amounts of AAV DNA synthesized at each adenovirus multiplicity were fairly comparable with either the WT or ts strains. This is in contrast to the restriction of adenovirus DNA synthesis with the ts mutants. Figure 2 is a plot of the ratios of labeled AAV DNA to labeled adenovirus DNA synthesized at each adenovirus multiplicity. It is evident that, at those mutant multiplicities for which there is a high probability of extensive dual infection with AAV, there is a significant dissociation between the ability to synthesize AAV DNA and adenovirus DNA. Furthermore, the small amounts of DNA synthesized by the mutants at the highest multiplicity were clearly not associated with an enhancement of AAV DNA synthesis. Thus, we conclude that the observed ability of the ts strains to support AAV DNA replication at 39.5 C is a function of their multiplicity (measured at 32 C) rather than their leakiness.

AAV DNA synthesis at 39.5 C was examined further at coninfection multiplicities which gave the largest relative dissociation with helper DNA synthesis in the previous experiment (2 mean tissue culture infective doses per cell for AAV and 5 mean tissue culture infective doses per cell for Ad5 strains). At 16 h after infection part of each culture was removed and labeled at 39.5 C with [^{3}H]thymidine for 1 h (the remain-

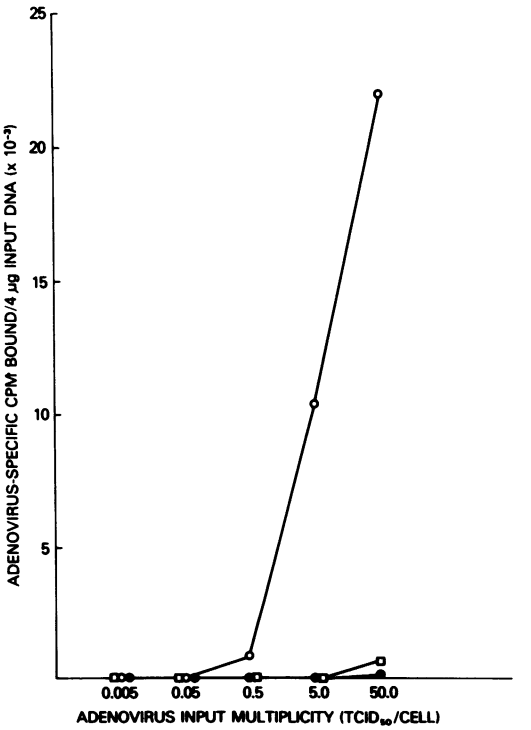

FIG. 1. *Synthesis of adenovirus-specific DNA in cultures infected with different multiplicities of Ad5 strains. DNA labeled with [^{3}H]thymidine from 10 to 20 h postinfection was hybridized with purified Ad5 DNA. Hybridized counts per minute of viral DNA in samples containing 4 μg of total extracted DNA are plotted for each input multiplicity of WT* (○), *ts125* (●), *and ts149* (□). *TCID$_{50}$, Mean tissue culture infective dose units.*

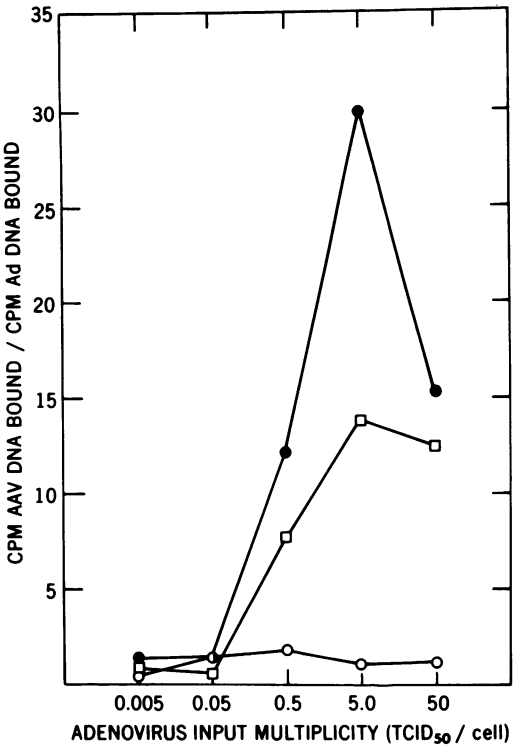

FIG. 2. *The ratios of AAV DNA counts per minute to adenovirus DNA counts per minute in 4-μg amounts of total DNA extracted from cultures coinfected with AAV-2 (2 $TCID_{50}$/cell) and each Ad5 strain at five different input multiplicities: AAV/WT (○), AAV/ts125 (●), and AAV/ts149 (□).*

der of each culture was used for experiments to be described). Total DNA was extracted from a portion of the labeled culture and hybridized as before (Table 3). These data again reveal that (i) AAV DNA synthesis does not detectably occur in the absence of helper, (ii) there is a marked restriction of adenovirus DNA synthesis by the ts mutants at 39.5 C, (iii) substantial amounts of AAV DNA are synthesized with any of the Ad5 strains, and (iv) there is no detectable cross-contamination of the adenovirus stocks with AAV. DNA from the remaining portions of the pulse-labeled cultures was also extracted by a modification of the Hirt method and analyzed by velocity sedimentation in neutral sucrose gradients. Figure 3A demonstrates the sedimentation pattern of labeled DNA from cells infected with the Ad5 WT strain alone. The counts coincide with the ^{32}P-labeled Ad5 marker DNA which was extracted from purified virions. Figure 3C shows the sedimentation pattern obtained from an extract of cells coinfected with AAV and WT. A peak coinciding with the Ad5 marker is present, but in addition there is a complex profile of DNA sedimenting between 10–21*S*. Recent work indicates that this material represents both replicating and mature AAV DNA molecules (E. D. Sebring, S. E. Straus, H. S. Ginsberg, and J. A. Rose, Fed. Proc., **34**:639, 1975). Figure 3B demonstrates the absence of detectable viral DNA in the Hirt extract from an infection with ts125 alone (a similar result was obtained with ts149 alone). Finally, Fig. 3D shows the gradient profile of DNA extracted from cells coinfected with AAV and ts125. As in Figure 3C, 10–21*S* material corresponding to AAV DNA is present, but in this case the 31*S* Ad5 DNA peak is absent (a similar result was obtained with ts149 as helper). The sedimentation profiles of AAV DNA synthesized with mutant helpers were indistinguishable from that synthesized with the WT adenovirus. It should be noted that essentially the same pattern shown in Figure 3C was observed when cells were coinfected at 32 C with AAV and with each of the three Ad5 strains. This series of experiments provides additional evidence that the ts mutants support AAV DNA replication at the nonpermissive temperature.

Viral RNA synthesis at 39.5 C. With the demonstration that AAV DNA synthesis is helped by the ts mutants it was important to determine whether the AAV genome is efficiently transcribed in the absence of detectable levels of adenovirus DNA synthesis and late mRNA transcription. In fact, the results of kinetic experiments exploring temporal associations between the appearance of adenovirus and AAV DNA and RNA have suggested that AAV transcription might depend on a factor(s) syn-

TABLE 3. *Virus-specific DNA synthesis in infections at 39.5 C*

Helper adenovirus	Without AAV coinfection		With AAV coinfection	
	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]
None	99(5)[b]	110(5)[b]	0(3)[c]	41(4)[c]
WT	27,709(5)	34(4)	16,033(5)	23,640(5)
ts125	131(7)	44(3)	77(3)	11,122(3)
ts149	1,101(3)	60(3)	826(4)	32,884(4)

[a] Expressed as total counts per minute bound per 4 μg of input DNA. Numbers in parentheses indicate the number of individual determinations averaged.

[b] Uninfected cells.

[c] Cells infected with AAV only.

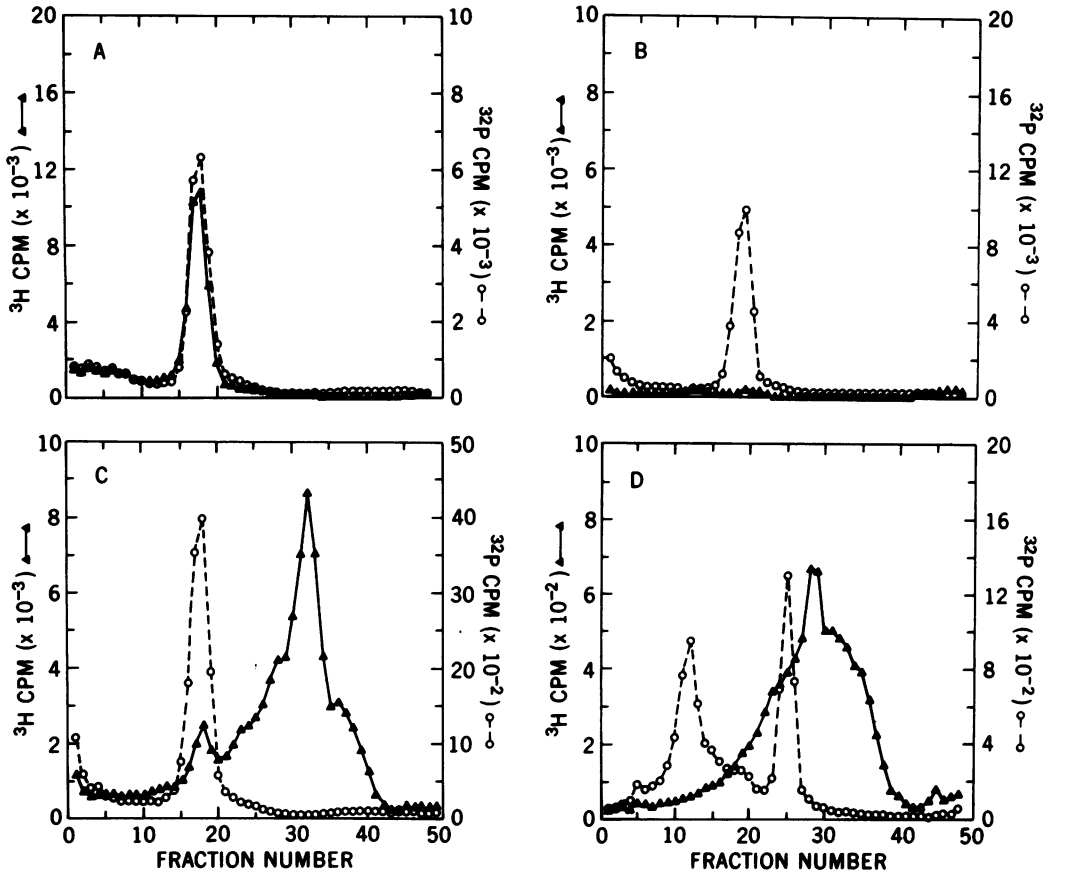

FIG. 3. *Velocity sedimentation in 5 to 30% neutral sucrose gradients of DNA extracted by the Hirt method from cultures infected with: (A) WT, (B) ts125, (C) WT + AAV, and (D) ts125 + AAV. Marker ^{32}P-labeled Ad5 DNA (31S) appears in all gradients, and ^{32}P-labeled simian virus 40 component II DNA (16S) is added to (D) only. Gradients (A), (B), and (C) were centrifuged at 40,000 rpm for 6 h and (D) was centrifuged at 40,000 rpm for 7.5 h.*

thesized late in the adenovirus cycle (6). Remaining portions of suspension cultures of coinfections with AAV and the Ad5 strains used in the previous experiment were labeled with [^{3}H]uridine for 1 h starting at 16 h after infection, and RNA was extracted and hybridized with either AAV or adenovirus DNA (Table 4). In agreement with data obtained at 37 C (19), AAV RNA was not synthesized at the nonpermissive temperature in the absence of adenovirus coinfection. AAV RNA, however, was synthesized in the presence of any of the Ad5 strains, and additionally, the amount of AAV RNA synthesized was comparable with each of the three Ad5 strains. (Again, as with DNA synthesis, quantitative comparisons of the RNA synthesis require the assumption that viral RNA specific activity is similar among the individual samples.) The relatively low levels of RNA synthesized by the mutants at 39.5 C presumably represent early messenger RNA (T. H. Carter and H. S. Ginsberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S 250, p. 255). This implies that the adenovirus helper function(s) needed for AAV transcription is independent of late adenovirus RNA synthesis.

Kinetics of viral nucleic acid synthesis at 39.5 C. Suspension cultures grown at 39.5 C were infected with AAV and with each of the Ad5 strains, and equal portions were labeled with [^{3}H]thymidine or [^{3}H]uridine for 1-h periods at various times after infection. As before, the labeled DNA and RNA were extracted and hybridized with either adenovirus or AAV DNA. Figure 4 shows plots of the synthesis of adenovirus and AAV DNA and RNA during each 1-h period. Panel A depicts kinetic relationships in the WT and AAV coinfection. These results are in excellent agreement with those previously reported using Ad2 as helper at 37 C (6). The

first detectable levels of adenovirus and AAV DNA occurred between 7 to 8 h after infection. Adenovirus RNA synthesis was observed between 3 to 5 h after infection and continued at low levels until 8 to 9 h when its synthetic rate increased sharply. Shortly after or coinciding with this increase in adenovirus RNA synthesis AAV RNA synthesis was first detected.

Panel 4B demonstrates kinetic relationships for the ts125 and AAV coinfection. Adenovirus DNA synthesis could not be detected until 10 to 11 h after infection when a small amount was found, and adenovirus RNA synthesis remained low without exhibiting the late rise seen with the WT (panel A). Despite the dramatic restriction in adenovirus DNA and RNA synthesis, the sequential appearance of AAV DNA and RNA was similar to that seen with the WT helper. Under restrictive conditions, then, not only is the synthesis of AAV DNA and RNA efficiently helped by the DNA-minus ts mutants, but (at least with helper ts125) there is still a lag in the appearance of AAV RNA synthesis.

TABLE 4. *Virus-specific RNA synthesis in infections at 39.5 C*

Helper adenovirus	Without AAV coinfection		With AAV coinfection	
	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]	Counts/min bound to Ad DNA[a]	Counts/min bound to AAV DNA[a]
None	40(2)[b]	158(2)[b]	0(3)[c]	51(3)[c]
WT	30,486(2)	129(2)	17,840(2)	17,201(2)
ts125	1,448(3)	36(2)	49(2)	14,564(2)
ts149	1,040(3)	0(2)	252(2)	20,356(2)

[a] Expressed as total counts per minute bound per 8 μg of input RNA. Numbers in parentheses indicate the number of individual determinations averaged.

[b] Uninfected cells.

[c] Cells infected with AAV only.

Synthesis of infectious virus at 39.5 C. In view of the incomplete helper activity of herpesviruses which permits AAV DNA and RNA synthesis but not infectious virion production (1, 3, 19), it was important to establish whether the DNA-minus mutants were complete helpers. Samples were taken from infected suspension cultures at 1 and 40 h after infection, and infectivity assays were carried out at 32 and 39.5 C. Table 5 shows titers of samples taken from coinfections which were used for experiments summarized in Tables 3 and 4. With WT or either mutant as helpers, a 2-log or greater increase in AAV titer occurred. Although the growth of WT increased about 3 logs, there was no significant rise in titer of either mutant at

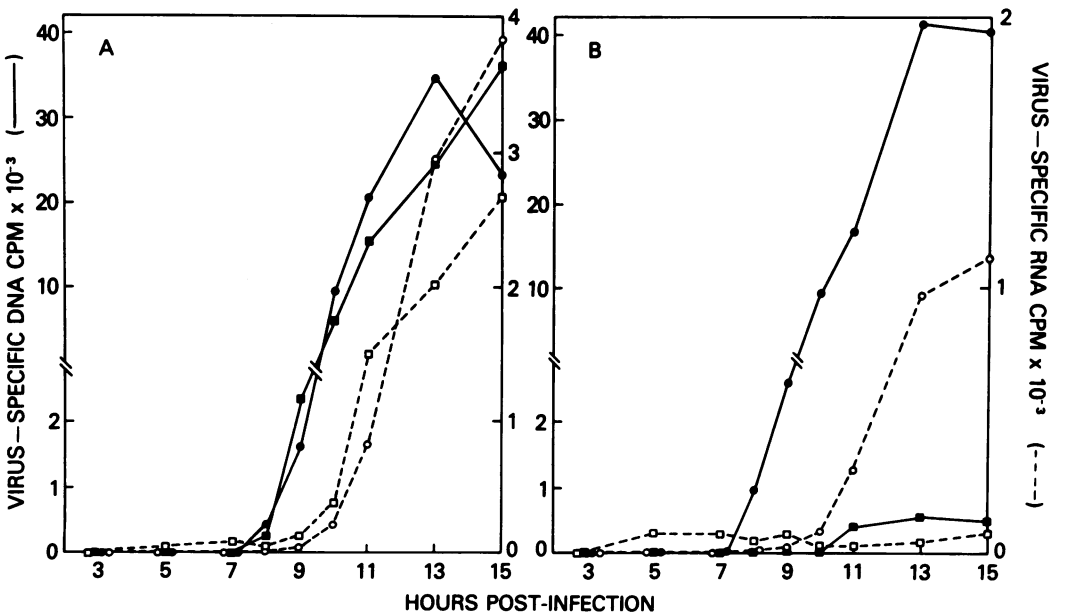

FIG. 4. *Kinetics of viral DNA and RNA synthesis at 39.5 C. Viral DNA and RNA synthesized during 1-h pulse periods at various times after infection are plotted as hybridizable counts per minute in 4-μg (DNA) or 8-μg (RNA) samples. (A) coinfection with WT (5 $TCID_{50}$/cell) and AAV-2 (2 $TCID_{50}$/cell), and (B) coinfection with ts125 (5 $TCID_{50}$/cell) and AAV-2 (2 $TCID_{50}$/cell). Adenovirus DNA (■), adenovirus RNA (□), AAV DNA (●), and AAV RNA (○).*

the nonpermissive temperature. Similar results were obtained with titers taken at 1 and 40 h after infection in which Ad5 strains were added in log increments (Fig. 1 and 2). These data (Table 6) demonstrate that, regardless of added multiplicity of the Ad5 mutant strains, there was no increase in titer of ts125 or t2149. In addition, they support an earlier observation that coinfection with AAV may suppress the growth of WT strains of adenoviruses (10). This in turn might relate to a suppression of adenovirus DNA synthesis which is apparent in Table 3 and Fig. 3C. Production of infectious AAV virions with the mutants closely paralleled that found with the helper WT at all multiplicites. Although it cannot be determined from these results whether there are significant differences in helper efficiency among the Ad5 strains, it is clear that the two Ad5 DNA-minus mutants are complete helpers of AAV multiplication at the nonpermissive temperature.

TABLE 5. *Viral yields of cultures infected at 39.5 C*[a]

Infection	1-h postinfection		40-h postinfection	
	Ad[b]	AAV[c]	Ad[b]	AAV[c]
AAV	0	3×10^4	0	3×10^8
WT	6×10^4	0	4×10^7	0
AAV + WT	2×10^4	1×10^4	3×10^7	1×10^8
ts125	2×10^4	0	1×10^4	0
AAV + ts125	3×10^4	1×10^5	1×10^5	1×10^7
ts149	3×10^4	0	1×10^4	0
AAV + ts149	1×10^5	3×10^8	1×10^4	3×10^6

[a] Titers as mean tissue culture infective dose units per milliliter.
[b] Ad titers at 32 C.
[c] AAV titers at 37 C.

DISCUSSION

Potentiation of AAV replication by an adenovirus DNA-minus ts mutant has been reported previously. Using a hemagglutination assay for AAV, Ito and Suzuki found AAV helper activity with the ts13 mutant of Ad31 (11). This result was corroborated by Mayor and Ratner who detected AAV replication by specific immunofluorescence (15). In the present study the ability of two Ad5 DNA-minus ts mutants to serve as AAV helpers was confirmed. Furthermore, an analysis of viral nucleic acid synthesis was carried out to help exclude mutant leakiness as the basis for AAV replication and to provide information concerning interrelationships of viral DNA and RNA synthesis. These data strongly indicate that mutant leakiness was not responsible for the observed helper activities of ts125 or ts149, and that the synthesis of AAV DNA and RNA proceeds normally with the mutant helpers. Thus, the mutants do

TABLE 6. *Titers of infections at 39.5 C*[a]

Adenovirus input multiplicity	Helper adenovirus	Without AAV coinfection		With AAV coinfection			
		Ad titer		Ad titer		AAV titer	
		1 h	40 h	1 h	40 h	1 h	40 hr
50.0	WT	$10^{4.0}$	$10^{8.5}$	$10^{4.5}$	$10^{5.5}$	$10^{5.5}$	$10^{7.5}$
	ts125	$10^{4.0}$	$10^{4.0}$	$10^{4.0}$	$10^{4.0}$	$10^{4.5}$	$10^{7.0}$
	ts149	ND	ND	$10^{5.0}$	$10^{4.0}$	$10^{4.5}$	$10^{6.5}$
5.0	WT	$10^{3.5}$	$10^{8.5}$	$10^{3.5}$	$10^{6.5}$	$10^{5.5}$	$10^{7.5}$
	ts125	$10^{3.5}$	$10^{4.0}$	$10^{4.0}$	$10^{3.0}$	$10^{4.5}$	$10^{6.5}$
	ts149	ND	ND	$10^{3.5}$	$10^{3.0}$	$10^{4.0}$	$10^{6.5}$
0.5	WT	$10^{2.5}$	$10^{5.5}$	$10^{2.5}$	$10^{3.5}$	$10^{5.5}$	$10^{6.5}$
	ts125	$<10^{2.0}$[b]	$10^{3.0}$	$10^{2.5}$	$<10^{2.0}$	$10^{5.0}$	$10^{6.5}$
	ts149	ND	ND	$10^{3.0}$	$10^{2.0}$	$10^{5.5}$	$10^{7.5}$
0.05	WT	$10^{2.5}$	$10^{3.0}$	$<10^{2.0}$	$10^{2.0}$	$10^{5.5}$	$10^{6.5}$
	ts125	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.0}$	$10^{5.5}$	$10^{6.0}$
	ts149	ND	ND	$10^{2.5}$	$10^{2.0}$	$10^{4.5}$	$10^{6.0}$
0.005	WT	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.5}$	$10^{5.5}$	$10^{5.5}$
	ts125	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.0}$	$<10^{2.0}$	$10^{5.0}$	$10^{5.0}$
	ts149	ND	ND	$<10^{2.0}$	$10^{2.0}$	$10^{4.5}$	$10^{4.5}$

[a] Titers as mean tissue culture infective dose units per milliliter. ND, Not done.
[b] Titer less than $10^{2.0}$ mean tissue culture infective dose units per milliliter.

not induce greater or lesser amounts of AAV DNA or RNA than WT, a conclusion that cannot be made unequivocally on the basis of titration data alone. The necessity for specifically defining the effects of all helpers on AAV macromolecular synthesis lies in the fact that little is known concerning the actual mechanism(s) involved in AAV enhancement.

The ability of adenovirus DNA-minus mutants to help AAV indicates that all of the genes directly required for adenovirus DNA replication would not be needed for AAV synthesis. This assumes, however, that AAV cannot utilize defective adenovirus proteins. Although it is possible that AAV multiplication does not depend on any function directly involved in adenovirus DNA synthesis, one would suspect that some gene product(s) required for adenovirus DNA synthesis might also be needed for AAV DNA replication, since both viral genomes contain a unique type of terminal repetition (8, 12, 25).

One puzzling feature concerning the ability of the adenovirus DNA-minus mutants to support AAV multiplication arises from previous kinetic data (5) as well as from the kinetic experiment described in the present study. The observed lag in initiation of AAV transcription after the onset of AAV DNA synthesis (Fig. 4A; 5), a lag which is abolished if cells are first preinfected with adenovirus for 10 h (5), suggests that AAV RNA synthesis may have to wait upon a factor(s) which is synthesized later in the adenovirus cycle. The apparent transcription delay also is clearly seen with ts125 at 39.5 C (Fig. 4B). The probability must therefore be considered that AAV transcription actually depends on an early cycle factor(s) whose action, for example, might be delayed until a critical concentration is reached. This substance could even represent an induced host cell factor. Alternatively, AAV transcription may not directly require any helper function, in which case an explanation for the observed lag is not readily apparent. Finally, there is an unlikely possibility that the helper DNA minus mutants are expressing late genes but at very low transcriptional levels, levels which provide the necessary catalytic amounts of one or more helper functions needed to complete AAV replication.

The precise biochemical defect(s) in any of the adenovirus DNA-minus mutants which have been shown to help AAV is not definitely known. Ad31 ts13 synthesizes T antigen, stimulates DNA polymerase and thymidine kinase, and suppresses host DNA synthesis at the nonpermissive temperature (21). Preliminary work with ts125 and ts149 has suggested that the temperature-sensitive defect may be linked to the initiation of DNA replication, but a defect in termination of DNA synthesis has not been excluded (H. S. Ginsberg, unpublished data). Two DNA binding proteins detectable after infection with Ad5 have been partially characterized by van der Vliet and Levine (22) and van der Vliet et al. (23). At the nonpermissive temperature little, if any, of either protein can be detected in cells infected with ts125, whereas both are present at normal levels in cells infected with either WT or Ad5 ts36 (a DNA-minus mutant which falls into the same complementation group at ts149). It thus seems unlikely that the binding proteins are specifically required for AAV replication. Furthermore, attempts to propagate AAV in cells transformed by Ad12 (10) or Ad2 (J. A. Rose, unpublished data) have failed. Since both transformed lines produce T antigen it would appear that this protein(s) by itself is incapable of supporting AAV multiplication. In addition, neither AAV DNA or RNA synthesis could be detected after infection of the Ad2-transformed line.

Although the collective data now argue that the necessary adenovirus helper activity is supplied early in its replicative cycle, perhaps after T antigen synthesis, it is not yet possible to completely rule out the need by AAV for an additional helper function normally expressed later in the helper virus cycle. In this regard it should be noted that herpes simplex virus induces AAV DNA and RNA (19) and protein synthesis (1), but infectious virions are not assembled (1, 3). The induced DNA has been reported to be infectious (4), and RNA molecules are similar to those found in adenovirus coinfections (6). Whether these findings reflect the absence of some other needed factor(s), possibly related to AAV assembly, remains to be determined.

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