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Adenovirus Helper Function for Growth of Adeno-Associated Virus: Effect of Temperature-Sensitive Mutations in Adenovirus Early Gene Region 2

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Adeno-associated virus (AAV) grows efficiently only in cells that are also infected with an adenovirus (Ad). We employed Ad mutants to determine which genes may be required for the AAV helper function. Two mutants of Ad type 5 (Ad5), Ad5*ts*125 and Ad5*ts*107, with temperature-sensitive lesions in the E72 DNA-binding protein coded by the Ad early region 2, were deficient for AAV helper functions at the nonpermissive temperature (40°C). In contrast, Ad5*ts*149, with a temperature-sensitive lesion in the Ad early region 5, was an efficient helper of AAV at the nonpermissive temperature. In KB cells, with the Ad5*ts*125 or Ad5*ts*107 mutant as the helper, the accumulation of AAV capsid proteins and AAV particles was decreased by about two logs, whereas AAV DNA synthesis was decreased only severalfold. Cytoplasmic, polyadenylic acid-containing AAV RNA is composed of a set of overlapping, spliced RNAs having different 5′ start points. With the *ts*125 helper at 40°C there was a decreased accumulation of some but not all of these AAV RNAs. The Ad5 E72 protein may have an effect on transcription or more likely posttranscriptional processing of AAV RNA. These observations suggest additional pleiotropic effects of the multifunctional E72 protein and suggest further similarities in the actions of E72 and the simian virus 40 T-antigen.

It is now well established that the defective parvovirus, adeno-associated virus (AAV), grows efficiently only in cells in which certain adenovirus (Ad) genes are being expressed. Thus, AAV may be useful as in independent probe for the analysis of certain Ad gene functions.

Several mutants of Ad serotype 5, 12, or 31 (Ad5, Ad12, Ad31), containing conditional-lethal, temperature-sensitive lesions in early genes that caused a DNA-negative phenotype at the restrictive temperature, were reported to help AAV efficiently (25, 28, 29, 42, 51). This suggested that Ad late functions were not required by AAV. However, there is one report (43) that one temperature-sensitive mutant of Ad31, which is unmapped but behaves phenotypically as a DNA-positive late mutant (52), was unable to promote AAV particle assembly, although AAV DNA and capsid antigens apparently were synthesized. In contrast, recent experiments show that purified Ad type 2 (Ad2) early mRNA which was microinjected into permissive cells provided the complete helper functions, sufficient for the growth of infectious AAV particles (49). When rodent cells transformed with the left-hand 16% of the Ad12 genome were infected with AAV, infectious AAV progeny were obtained if the cells were subsequently fused with permissive human (KB) cells (25). This suggested that, at least for AD12, only a subset of early genes was required for the AAV helper function. In contrast, AAV does not grow in human 293 cells transformed with the left-hand 16% of the Ad5 genome (C. Laughlin, N. Jones, T. Shenk, and B. J. Carter, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, S 160, p. 266). Clearly, further understanding of the biochemical basis of the Ad helper function(s) for AAV would be aided by the availability of precisely mapped Ad mutants which are defective for some aspect of AAV growth.

The Ad5 early genes are located in at least five clearly separated regions of the Ad genome. These are referred to as early regions 1 through 4 (for review, see reference 16), and the most recently described region (20) is referred to as region 5.

In this report, we investigated the replication of AAV in human (KB) cells at the restrictive temperature (40°C) with Ad5 mutants with temperature-sensitive lesions in early genes. In contrast to previous reports by others (24, 51), we show that Ad5 mutants containing conditional-lethal, temperature-sensitive lesions in early region 2 are much less efficient for some AAV helper functions than is wild-type Ad. These

studies suggest additional pleiotropic effects of the E72 DNA-binding protein coded for by the Ad early region 2. Thus, AAV may offer a useful independent probe to separately analyze certain of the pleiotropic properties of the Ad E72 protein.

MATERIALS AND METHODS

Viruses and cells. Stocks of AAV type 2 (AAV2), wild-type Ad2 (Ad2wt), or wild-type Ad5 (Ad5wt) were grown in KB-3 cells in a suspension culture at 37°C. Stocks of Ad temperature-sensitive mutants were grown in KB cells at 32°C. All Ad stocks were assayed by plaque formation at 32 and 40°C on KB cells in a monolayer culture. AAV stocks were assayed in KB monolayers with the fluorescent nuclei assay, as described previously (10).

The Ad2wt virus was that used previously and originated from the laboratory of W. Rowe (National Institutes of Health). The Ad5*ts*125 mutant and the parent strain Ad5wt were those described by Ensinger and Ginsberg (14) and were obtained from A. Levine (Princeton University). Ad5*ts*107 (22) and Ad5*ts*149 (14) were obtained from H. Young and H. Ginsberg (Columbia University), and Ad2*ts*215 was obtained from W. Doerfler (Cologne). For all experiments, the permissive temperature was 32°C, and the nonpermissive temperature was 40°C. Care was taken to ensure that the nonpermissive temperature did not fall below 40°C. The ratio of the infectious titer obtained by plaque formation on KB monolayer cells at the permissive temperature relative to the nonpermissive temperature was greater than 10^5 for all the mutants. For wild-type Ad, this ratio was approximately 1.0.

In all the experiments reported here, KB cells were grown in monolayer culture in Eagle medium containing 10% fetal calf serum. Cells were routinely monitored with the Hoechst staining procedure (12) to ensure the absence of mycoplasma contamination. Cells were grown to 60 to 80% confluency and then infected with AAV and Ad. Adsorption of virus was performed in phosphate-buffered saline (pH 7.2) containing 2% fetal calf serum for 60 to 90 min at room temperature. The inoculum was removed, and the cells were rinsed once with Eagle medium and incubated in fresh medium containing 2% fetal calf serum.

Measurement of viral yield by fluorescent staining of cell nuclei or by an infectivity assay. KB cells were grown on microscopic slides equipped with chambers (Lab-Tek Products, Div. Miles Laboratories Inc., Napierville, Ill.) with 10^5 cells per chamber. Cells were infected with AAV and Ad as described above. For determination of the proportion of nuclei that were fluorescent for AVV or Ad antigens, cells were fixed at 30 h after infection when grown at 37 or 40°C or at 50 h after infection when grown at 32°C. Cells were stained with a double antibody procedure described previously (10). The first antibody was either rabbit immunoglobulin G directed against AAV2 particles or rabbit antiserum directed against Ad2 or Ad5 particles. The second antibody was FITC-conjugated hamster immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) directed against rabbit immunoglobulin G.

For measurement of infectious yields of AAV, the infected cultures were grown for 48 h at 37 and 40°C or for 96 h at 32°C and then frozen and thawed three times.

Analysis of virus particles. Both the DNA and protein components of virus particles were labeled by incubating cultures in Eagle medium containing methionine at 1/10 the normal concentration (i.e., at 0.3 μg/ml) and supplemented with [^{35}S]methionine (specific activity, 900 to 1,100 Ci/mmol) and [^{3}H]thymidine (specific activity, 40 to 60 Ci/mmol) at a final concentration of 20 to 40 μCi/ml. Virus particles were analyzed by a procedure described in detail elsewhere (44). Briefly, cells were harvested at 30 h after infection, frozen and thawed in TE buffer (50 mM Tris-hydrochloride [pH 8.7]–0.5 mM EDTA), sonicated, and treated with *n*-butanol (1% final concentration) at 4°C for 1 h. The lysate was banded to equilibrium in a CsCl gradient in an SW41 rotor of a Beckman centrifuge. Fractions from the density gradient were pooled into five contiguous regions, in grams per cubic centimeter, as follows: 1.310 to 1.338, 1.340 to 1.360, 1.361 to 1.390, 1.391 to 1.435, and 1.436 to 1.485. These density regions are referred to, for ease of identification, as 1.32, 1.35, 1.37, 1.41, and 1.45, respectively. Portions of each region were dialyzed and sedimented in neutral sucrose velocity gradients.

Analysis of intracellular viral DNA. Intracellular viral DNA was labeled with [^{3}H]thymidine as described above and selectively extracted with a modified Hirt (27) procedure as described elsewhere (10). The extracted viral DNA was analyzed by sedimentation in 5 to 20% sucrose gradients containing 1 M NaCl, 50 mM Tris-hydrochloride (pH 8.0), 0.15% Sarkosyl, and 1 mM EDTA at 4°C for 16 h at 28,000 rpm.

Analysis of intracellular viral proteins. Infected cell proteins were labeled with [^{35}S]methionine (50 μCi/ml) from 12 to 30 h after infection. Cells were lysed in Tris-buffered saline containing 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 10 mM 2-mercaptoethanol and centrifuged for 15 min at 16,000 × *g*. The supernatant was diluted 10-fold with Tris-buffered saline and reacted with normal rabbit serum or the immunoglobulin G fraction of rabbit serum prepared against purified AAV2 particles. Immunoprecipitates were then collected (32) and solubilized in sodium dodecyl sulfate buffer and electrophoresed in 9% acrylamide gels (38, 46).

Preparation of RNA. KB cells were coinfected with AAV2 at a multiplicity of 20 infectious units (IU) per cell and either Ad5wt or Ad5*ts*125 at a multiplicity of 5 PFU per cell. Infected cultures were grown at 40°C, and cytoplasmic RNA was prepared at 18 h after infection as described previously (40). Polyadenylic acid-containing RNA was selected with columns of oligo(dT)-cellulose.

Analysis of DNA-RNA hybrids by gel electrophoresis. AAV RNA was annealed to AAV DNA, and the DNA-RNA hybrids were analyzed with the procedure of Berk and Sharp (2, 3). Briefly, RNA preparations were mixed with duplex AAV [^{32}P]DNA (10^6 to 2×10^6 cpm per μg) and then heat denatured and annealed in an 80% formamide solution at 48°C for 2 h. The resultant DNA-RNA hybrids were digested with endonuclease S1 (EC 3.1.30.1) from *Aspergillus*

oryzae (Miles Laboratories) and then electrophoresed in 1.4% agarose gels at neutral pH. Gels were dried and visualized by fluorography (6) with a Dupont Cronex Lightning plus intensifying screen and SB5 Kodak film at −70°C. Annealing reactions were performed with the DNA present in significant excess.

RESULTS

Properties of Ad mutants. The Ad5 mutants Ad5*ts*125 and Ad5*ts*107 are independently isolated strains (14, 21, 22) having a temperature-sensitive lesion, presumably a point mutation, located approximately between map coordinates 62 and 64 (on a scale of 100 U for the whole Ad genome) within the Ad early gene region 2 (19). This region of Ad codes for a 72,000-molecular-weight DNA-binding protein, E72 (53), that is synthesized early after infection before the onset of viral DNA synthesis. These mutants produce an E72 protein that, at the nonpermissive temperature, is thermolabile in its ability to bind to single- or double-stranded DNA (17, 54). The mutant Ad5*ts*149, (14, 21) has recently been located by marker rescue on the Ad5 genome in early region 5 between map coordinates 18 and 22. All three mutants, Ad5*ts*125, Ad5*ts*107, and Ad5*ts*149, exhibit a DNA-negative phenotype at the nonpermissive temperature. Ad5*ts*149 was reported to be defective for cellular transformation, whereas Ad5*ts*125 and Ad5*ts*107 produced increased efficiency of cellular transformation at the nonpermissive temperature (21).

The Ad2 mutant Ad2*ts*215 is a previously undescribed and unmapped mutant which appears to be defective for some late function required for Ad assembly (44).

Growth of AAV with Ad5*ts*125 as the helper. The growth of AAV at 32 and 40°C was measured by the appearance of AAV capsid antigens in the infected cell nuclei (Fig. 1). At 40°C with Ad5wt as the helper, the accumulation of AAV antigen began at about 12 h and was completed by about 30 h after infection. This time scale is very similar to that observed at 37°C (data not shown). At 32°C, the growth of AAV was slowed by about twofold. When the mutant Ad5*ts*125 was used as a helper for AAV, it was as efficient as Ad5wt at 32°C but was very inefficient at 40°C (Fig. 1). Based on these data, subsequent analyses of AAV antigen production were performed by fixing cells at 30 h after infection at 37 or 40°C or at 50 to 60 h after infection at 32°C.

In additional experiments, both AAV antigens and AAV infectivity were assayed (Table 1). Both Ad5wt and the late mutant Ad2*ts*215 helped AAV efficiently at 32, 37, or 40°C. In contrast, when Ad5*ts*125 was used at 40°C, the yields of AAV antigen and AAV infectivity were decreased by about two logs. This decrease paralleled the decrease in production of Ad structural antigens. At 32 or 37°C, the Ad5*ts*125 mutant behaved as a wild type for both Ad and

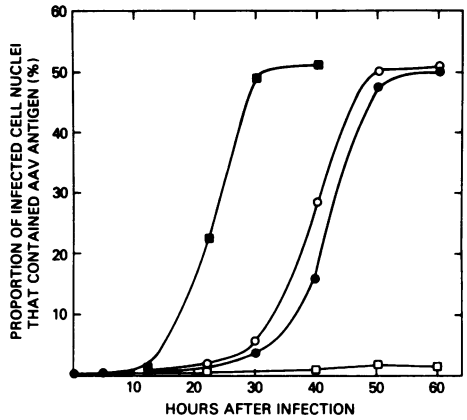

FIG. 1. *Appearance of AAV antigens in the nuclei of infected cells. KB monolayer cells were infected with AAV (10 IU per cell) and either Ad5wt or Ad5ts125 helper virus (5 PFU per cell) and then grown at 40 or 32°C. At the times indicated, cells were fixed and stained with fluorescent antibody to detect AAV antigens. The results show the proportion of fluorescently stained nuclei observed. Symbols:* ■—■, *40°C, AAV plus Ad5wt;* □—□, *40°C, AAV plus Adts125;* ●—●, *32°C, AAV plus Ad5wt;* ○—○, *32C, AAV plus Adts125.*

TABLE 1. *Assay of AAV antigens and AAV infectivity*[a]

Ad helper	Temp (°C)	% of cell nuclei fluorescent for:		Yield of infectious AAV particles (AAV per cell [×10⁻⁴])
		Ad	AAV	
Ad5*ts*125	32	100	55	1.5
	37	90	48	2.5
	40	2	0.5	0.01
Ad2*ts*215	32	100	45	1.5
	37	100	35	2.0
	40	100	51	1.25
Ad5wt	32	100	47	1.0
	37	100	41	1.0
	40	100	49	1.0
None	40	0	0	0.0001

[a] KB monolayer cells were infected with AAV2 (10 IU per cell) and Ad (10 PFU per cell) and grown at the temperatures shown. Cells were either fixed and assayed by immunofluorescence for Ad or AAV capsid antigens or lysed and assayed for AAV infectivity. The infectivity data show the number of IU obtained per cell.

AAV growth. Further analyses (Table 2) showed that the inability of Ad5*ts*125 to help AAV at 40°C could not be overcome by a high multiplicity of infection with the mutant. These results show clearly that the Ad5*ts*125 mutant is a deficient helper for AAV at the nonpermissive temperature. This is not, however, a general property of Ad temperature-sensitive mutants, as indicated by the efficient helper function provided by Ad2*ts*215.

At the permissive temperature (or nonpermissive temperature with Ad2wt), nearly all the cells were successfully infected with Ad, as judged by fluorescent antibody staining for Ad antigens (Tables 1 and 2). However, only about one-half of the cell nuclei showed fluorescence when stained for AAV capsid antigens. This phenomenon is not clearly understood but is routinely observed in cells infected with AAV and Ad (10).

Growth of AAV with Ad5*ts*107 or Ad5*ts*149 as the helper. The failure of Ad5*ts*125 to help AAV might be related specifically to the mutation in E72, or it might be a general property of Ad DNA-negative mutants. To answer these questions, we used both Ad5*ts*107, which does not contain the second site mutation present in Ad5*ts*125, and Ad5*ts*149, which maps in Ad early region 5. As shown in Table 3, the Ad5*ts*107 mutant was also deficient for AAV helper function at 40°C. In contrast, the Ad5*ts*149 mutant helped AAV efficiently at all temperatures. Thus, the inability to help AAV appears to be a discrete property of the Ad5*ts*125 and Ad5*ts*107 mutations in the Ad E72 protein.

Effect of a temperature shift on the AAV helper function provided by Ad5*ts*125. Figure 2 shows the results of an experiment in which cells were infected with AAV and Ad5*ts*125 and grown at 32°C for varying periods of time before shifting to 40°C. These results

TABLE 2. *Effect of helper multiplicity on the growth of AAV at the nonpermissive temperature*[a]

Ad helper	Helper multiplicity (PFU per cell)	% of cell nuclei fluorescent for:	
		Ad	AAV
Ad5wt	25	100	60
	2.5	81	31
	0.25	20	11
Ad5*ts*125	200	0	3
	20	0	0
	2	0	0

[a] KB monolayer cells were infected with AAV2 (10 IU per cell) and helper Ad as indicated and grown at 40°C. Cells were fixed and stained for AAV or Ad antigens at 30 h after infection.

TABLE 3. *Growth of AAV with Ad temperature-sensitive, DNA-negative mutants*[a]

Ad helper	Helper multiplicity (PFU per cell)	Temp °C	% of cell nuclei fluorescent for:		Yield of infectious AAV particles (AAV per cell [$\times 10^{-4}$])
			Ad	AAV	
Ad5*ts*107	40	32	85	56	1.7
		37	56	36	0.5
		40	0	0	0.01
Ad5*ts*107	4	32	73	52	
		37	7	5	
		40	1	0	
Ad5*ts*107	0.4	32	33	21	
		37	1	0	
		40	0	0	
Ad5*ts*149	100	32	86	59	
		37	42	42	
		40	0	50	
Ad5*ts*149	10	32	83	53	3.0
		37	25	41	
		40	0	49	2.0
Ad5*ts*149	1	32	43	29	
		37	16	24	
		40	0	17	
None	0	40	0	0	0.0005

[a] Cells were infected with AAV2 at a multiplicity of 15 IU per cell and Ad as indicated. All procedures were as for Table 1.

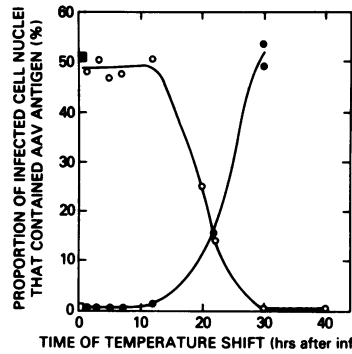

FIG. 2. *Effect of temperature shift on the function of Ad5ts125 as a helper virus for AAV. Two sets of KB monolayer cell cultures were infected with AAV2 (10 IU per cell) and Adts125 (5 PFU per cell). One set of cultures* (○——○) *was infected and grown at 40°C, and at the times indicated, individual cultures were shifted to 32°C, and growth was continued. All cultures in this set were fixed at 50 h after infection and then stained for nuclear accumulation of AAV capsid antigens. The other set of cultures* (●——●) *was grown at 32°C, and individual cultures were shifted to 40°C at the times indicated. All cultures in this set were fixed for staining at 38 h after infection. Other control infected cultures were maintained continuously at 40°C and fixed at 38 h after infection* (□) *or at 32°C and fixed at 50 h after infection* (■).

suggest that at 32°C the AAV helper function appeared between 10 and 30 h after infection. These times are equivalent to about 5 to 15 h after infection at 37 or 40°C if the growth curves of Fig. 1 are considered. In the converse experiment, cells were first held at 40°C, and then shifted to 32°C. As the period at 40°C was extended for longer than 10 h, there was a progressive decrease in the yield of AAV when it was assayed at 50 h. This suggests that the Ad helper function could not be accumulated in sufficient quantity at 40°C. These observations support the role of E72 in the AAV helper function and are consistent with the known properties of this protein (see Discussion).

Synthesis of intracellular AAV DNA. The above experiments showed that at the nonpermissive temperature there was a decrease in production of infectious AAV by about two logs when the Ad mutants Ad5*ts*125 or Ad5*ts*107 were used as helpers. To investigate further the biochemical defect, we measured the synthesis of intracellular AAV DNA at 40°C with either wild-type or mutant helpers (Fig. 3). Viral DNA was labeled with [^{3}H]thymidine and selectively extracted as described above. In the gradients of Fig. 3, all of the [^{3}H]DNA sedimenting more slowly than the 31S Ad DNA is AAV specific, as judged by resedimentation in neutral and alkaline sucrose gradients, restriction endonuclease cleavage (10, 39) and gel electrophoresis, and DNA-DNA hybridization (data not shown). When Ad5*ts*125 was the helper there was a fivefold decrease in the labeling of AAV DNA. The incorporation of [^{3}H]thymidine into both standard (14.5S) AAV DNA and slower-sedimenting (10S) AAV DNA was decreased to a similar extent (Fig. 3). The slower-sedimenting 10S DNA corresponds to a heterogeneous population of variant AAV DNA molecules (13, 26). At least some of these variant AAV molecules are defective-interfering genomes (10, 39). Figure 3 also shows that the incorporation of [^{3}H]thymidine into 31S Ad DNA is abolished when the Ad5*ts*125 mutant is used at 40°C. In similar experiments (not shown) performed at 40°C, AAV DNA synthesis with Ad5*ts*107 as the helper was identical to that with Ad5*ts*125, but with Ad5*ts*149 it was the same as for Ad5wt. Both Ad5*ts*107 and Ad5*ts*149 were negative for Ad DNA synthesis at 40°C. These results are qualitatively consistent with the biological assays described above. However, the fivefold decrease in AAV DNA synthesis does not directly account for the 100-fold decrease in the production of infectious AAV when either Ad5*ts*125 or Ad5*ts*107 was used as the helper at 40°C. Furthermore, the fivefold decrease in AAV DNA synthesis (Fig. 3) was the maximum observed when Ad5*ts*125 was used. In various experiments, the decrease ranged from two- to fivefold.

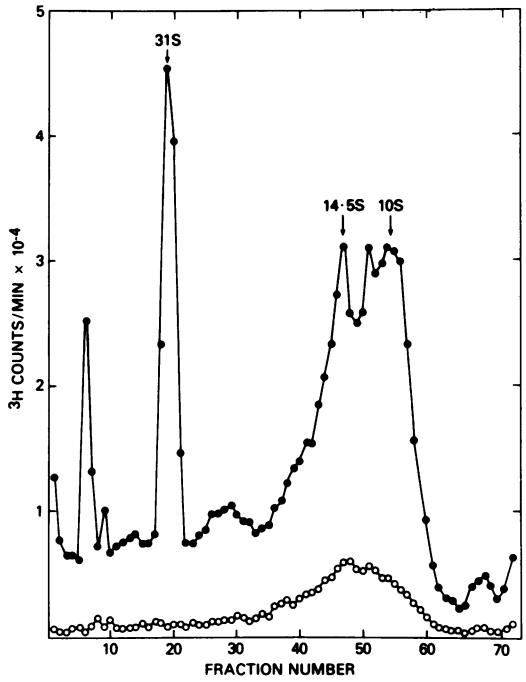

FIG. 3. *Analysis of viral DNA synthesis. Monolayer cultures (75-cm^2 T-flask) of KB cells were infected with AAV2 (10 IU per cell) and either Ad5wt* (●——●) *or Ad5ts125* (○——○) *as the helper virus (5 PFU per cell) and grown at 40°C. Viral DNA was labeled by the addition of [^{3}H]thymidine from 16 to 20 h after infection and then selectively extracted and sedimented in a neutral sucrose gradient. Sedimentation is from right to left.*

Formation of AAV particles. The assembly of AAV particles at 40°C in cells with either a wild-type or mutant helper was analyzed by labeling with both [^{3}H]thymidine and [^{35}S]methionine. The labeled AAV particles were then analyzed by fractionation isopycnic centrifugation in CsCl. The CsCl gradients were divided into several regions, and portions of each region were analyzed by velocity sedimentation in neutral sucrose gradients to resolve the various types of AAV particles. Figure 4 shows the velocity sedimentation analysis of selected regions characteristic of standard 110S AAV particles (density region, 1.41 g/cm^3; Fig. 4A), 82S AAV defective-interfering particles (density region, 1.37 g/cm^3; Fig. 4B), or 66S empty AAV particles (density region, 1.32 g/cm^3; Fig. 4C). In Fig. 4D, E, and F are shown the corresponding density regions from lysates of parallel cultures infected with wild-type or mutant Ad alone. The 1.37- and 1.32-g/cm^3 regions (Fig. 4E and F), respectively, contained some full or empty Ad particles

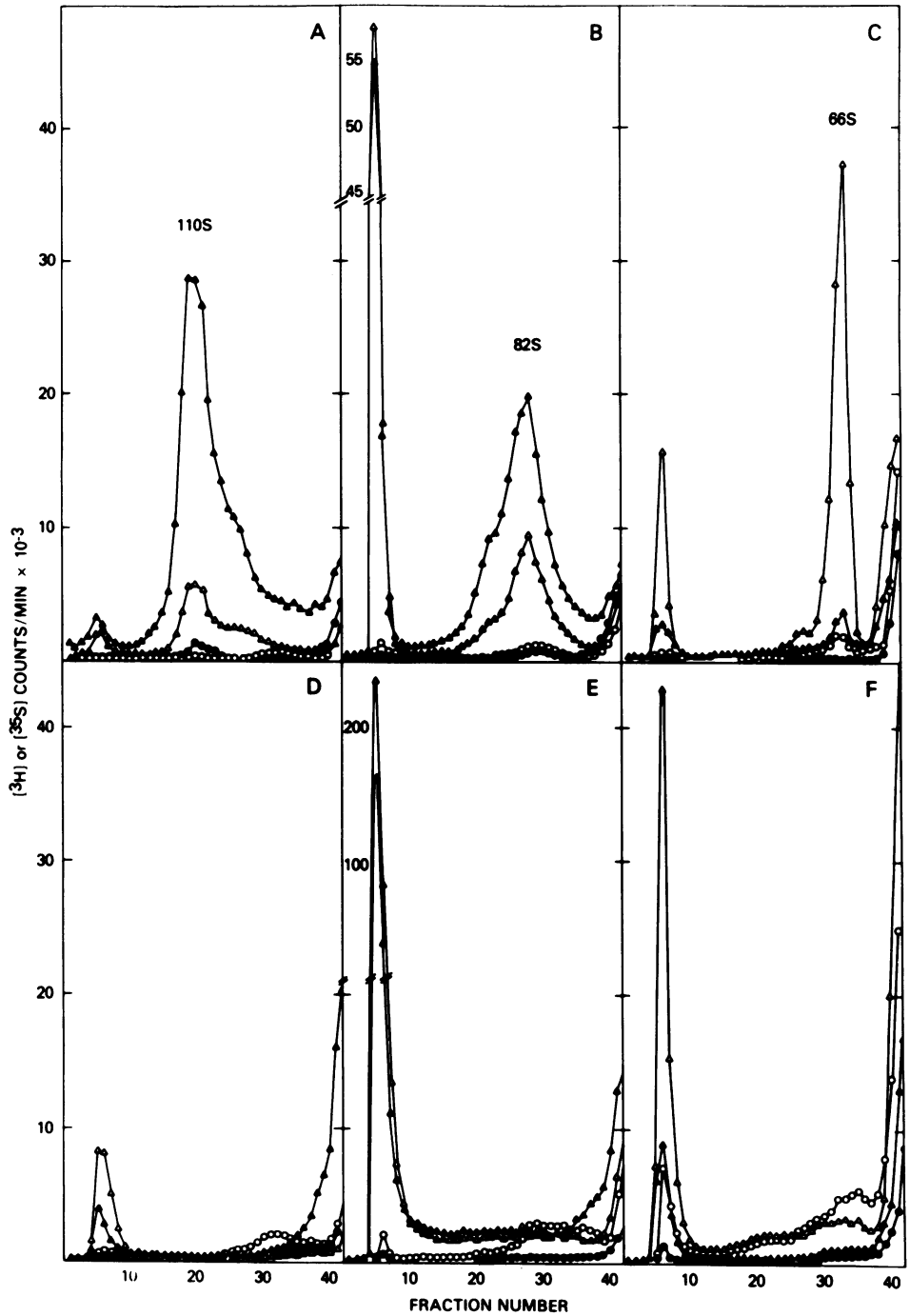

FIG. 4. *Synthesis of AAV particles at 40°C. Cultures (150 cm²) of KB monolayer cells were infected with AAV2 (10 IU per cell) together with Ad5wt or Ad5ts125 (5 PFU per cell). Additional cultures were infected with Ad5wt or Ad5ts125 in the absence of AAV. All the cultures were grown at 40°C, labeled with [³H]-thymidine and [³⁵S]methionine from 16 to 26 h after infection, and then lysed and fractionated in CsCl buoyant density gradients. Portions of each density region from the CsCl gradient were then sedimented in neutral sucrose gradients. Panels A, B, and C show the 1.41-, 1.37- and 1.32-g/cm³ density regions, respectively, from lysates of cells infected jointly with AAV and Ad. Panels D, E, and F show the analogous density regions from lysates of cells infected with Ad only. Symbols: ▲——▲, [³H]thymidine or △——△, [³⁵S]methionine from cultures in which Ad5wt was used; ●——●, [³H]thymidine or ○——○, [³⁵S]methionine from cultures in which Ad5ts125 was used. The sucrose gradients were formed over a shelf of CsCl (density, 1.71 g/cm³) (fractions 1 to 5 in panels A through F). Note the break in the scale in panels B and E.*

that sedimented onto the CsCl cushion beneath the sucrose gradient (Fig. 3, fraction 5). The bulk of mature Ad particles banded at 1.35 g/cm^3 (not shown). In cultures infected with Ad5*ts*125, the incorporation of both [^{3}H]thymidine and [^{35}S]-methionine into Ad particles was greatly decreased (at least 450-fold), as expected.

When Ad5*ts*125 was the helper at 40°C, the incorporation of [^{3}H]thymidine and [^{35}S]methionine into AAV particles of all density classes, including 1.41, 1.37, and 1.32 g/cm^3 (Fig. 4A, B, and C), was decreased at least 50- to 100-fold. Similar results (not shown) were obtained when the AAV (1.45-g/cm^3) and AAV (1.35-g/cm^3) particles were analyzed. This decrease of about two logs in the labeling of both empty or full AAV particles appears to account for most of the decrease in production of infectious AAV when Ad*ts*125 was the helper.

Synthesis of AAV capsid proteins. AAV particles contain three major polypeptides, VP1, VP2, and VP3 (previously labeled A, B, and C), with molecular weights of 85,000, 72,000, and 61,500, respectively (50). All three proteins are coded for by AAV mRNA (7). Synthesis of these proteins in infected cells at 40°C with a wild-type or mutant helper was examined as shown in Fig. 5. ^{35}S-labeled AAV proteins were immunoprecipitated from whole cell lysates with an anti-AAV2 antibody which is known to be able to precipitate all three AAV proteins after in vitro translation (F. Jay and B. Carter, unpublished data). Intracellular accumulation of all three AAV proteins was greatly decreased at 40°C when the Ad5*ts*125 mutant was the helper (Fig. 5, lane e). In the analysis of proteins from cells with the Ad5wt helper (Fig. 5, lane c) the AAV VP2 protein was partly obscured by a 73,000-molecular-weight protein. This protein, and also the 63,000-molecular-weight protein seen with the Ad5*ts*125 mutant helper (lanes d and e), precipitated under the conditions used with either anti-AAV2 antibody or normal rabbit serum. These proteins were also precipitated from cells infected, respectively, with Ad5wt or Ad5*ts*125 alone (not shown) and thus do not appear to be AAV specific.

Analysis of cytoplasmic AAV RNA. The structures and genome locations of the predominant polyadenylic acid-containing AAV RNAs previously obtained from the cytoplasm of KB cells in which wild-type Ad was the helper at 37°C are summarized in Fig. 6 (40). All of the RNAs are complementary to the AAV DNA minus strand. The RNAs *a*, *c*, *e*, and *g* have a spliced-out region with approximate map coordinates of 41/48. It is not yet known whether the four different 5′ termini at map positions 5, 13, 19, and 39/40 reflect differences in promoter sites or posttranscriptional processing.

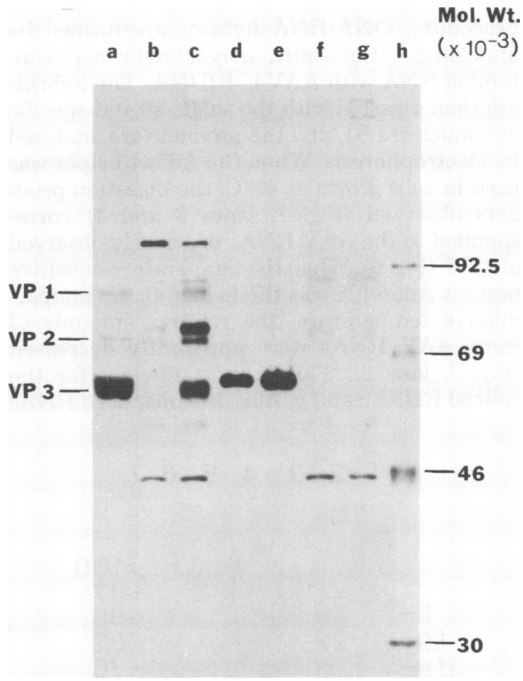

FIG. 5. *Electrophoresis of immunoprecipitated ^{35}S-labeled proteins obtained from KB cells infected at 40°C with AAV plus Ad5wt (lanes b and c), AAV plus Ad5ts125 (lanes d and e), or uninfected cells (lanes f and g). Immunoprecipitation was performed with normal rabbit serum (lanes b, d, and f) or anti-AAV2 immunoglobulin G (lanes c, e, and g). Markers are AAV proteins from purified [^{35}S]labeled virions (lane a) or [methyl-^{14}C]phosphorylase B, albumin, ovalbumin, or carbonic anhydrase (lane h).*

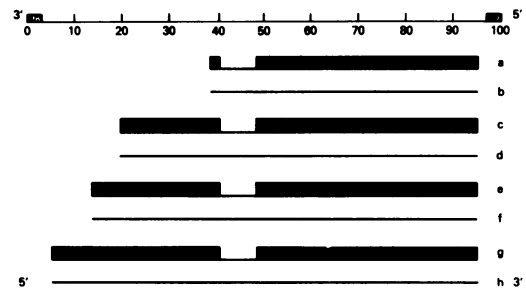

FIG. 6. *Schematic diagram of polyadenylic acid-containing cytoplasmic AAV RNAs. The upper line represents the AAV DNA minus strand. Stippled blocks indicate the terminal palindromic repetitions (4, 37). Lines a to h indicate the location and structure of AAV RNA species previously identified (40). RNAs a, c, e and g are spliced, with the solid boxes indicating the regions retained after splicing. RNAs b, d, f, and h are unspliced AAV RNAs.*

Cytoplasmic AAV RNA was analyzed by the procedure of Berk and Sharp (2, 3). In this

procedure, DNA-RNA hybrids are formed by annealing cytoplasmic, polyadenylic acid-containing RNA with AAV [^{32}P]DNA. The hybrids are then digested with the single-strand-specific endonuclease S1, and the products are analyzed by electrophoresis. When the Ad5wt helper was used in cells grown at 40°C, the digestion products observed (Fig. 7, lanes 3 and 4) corresponded to the AAV RNAs previously observed at 37°C (Fig. 6). When the temperature-sensitive mutant Ad5*ts*125 was the helper at the nonpermissive temperature, the relative amounts of some AAV RNAs were apparently decreased (Fig. 7, lane 2). This is most obvious for the spliced RNAs *c* and *g*. Also, the unspliced RNAs

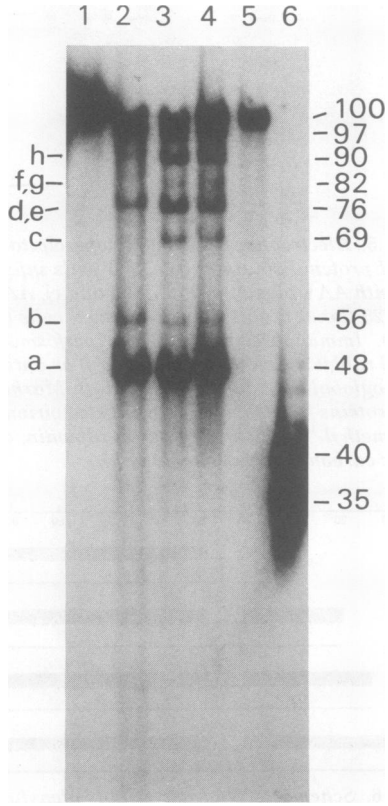

FIG. 7. *Analysis of AAV RNA (2, 3). AAV [^{32}P]-DNA was annealed with RNA from cells infected with AAV and Ad5ts125 (lane 2) or wild-type helper (lanes 3 and 4) or with yeast RNA (lane 5). For the reactions in lane 4, the digestion contained one-half of the amount of endonuclease S1 that was used in the other reactions. Lane 1, duplex AAV DNA; lane 6, AAV DNA HaeII fragments A and B. The individual digestion products are identified at the right by size (expressed as a percent of the size of the AAV DNA duplex) and at the left according to the RNA species (Fig. 6) from which they are derived.*

d and *h* may be reduced somewhat. In contrast, the amounts of RNAs *a* and *b* appeared to be similar with either helper. These conclusions were supported by additional autoradiographic analyses in which both the amount of RNA and the duration of autoradiography were varied to avoid possible darkening of the film beyond the linear exposure range. Rigorous confirmation of this point requires accurate titration of the various mRNA species. None of the RNAs *a* to *h* were observed when RNA from yeast (Fig. 7, lane 5) or cells infected only with Ad (40) was used. In RNA preparations from both mutant and wild-type helper cells, the 76% digestion product was derived from both the unspliced RNA *d* (the major component) and the spliced RNA *e* (the minor component). This was verified by additional analysis of the digestion products on alkaline agarose gels (not shown), which demonstrated both a 76% band from RNA *d* and a 27% band from the leader of RNA *e*. The main body of RNA *e* (46.5% band) cannot be distinguished from that of RNA *a*, *c*, or *g*. A similar analysis showed that most of the 82% band observed in Fig. 7 was derived from the spliced RNA *g*. In the analysis of Fig. 7, a band with an approximate size of 97% is present in the products obtained with RNA from cells jointly infected with AAV and Ad (Fig. 4, lanes 2, 3, and 4). This band is not always observed (40) but appears to be AAV specific and is never seen when RNA from yeast (Fig. 4, lane 5) or cells infected only with Ad is used. This product may represent a DNA-RNA hybrid derived from RNA *h*, in which the endonuclease S1 has not degraded the self-annealed palindromic terminal DNA sequences. This suggests that RNA *h* may extend over the entire region of the AAV genome between these palindromes, i.e., with coordinates 3/97. The band at 100% is derived from DNA-DNA annealing, as shown by its presence in the reactions with yeast RNA (Fig. 7, lane 5).

DISCUSSION

Our experiments show that in KB cells at the nonpermissive temperature (40°C), the Ad mutants Ad5*ts*125 and Ad5*ts*107 were deficient helpers of AAV. The synthesis of both infectious and noninfectious AAV particles, as well as the intracellular accumulation of AAV-specific immunofluorescence, were decreased by 50- to 200-fold. These phenomena were accounted for by the demonstration that the accumulation of the AAV capsid proteins VP1, VP2, and VP3 was greatly decreased with the mutant helpers.

With the mutant helpers, AAV DNA synthesis was decreased four- to fivefold, which does not directly account for the much greater de-

crease in accumulation of AAV particles. The E72 protein is required for Ad DNA replication (54) and is also involved in the negative regulation of its own, as well as other, Ad early mRNA's (5, 11). It is not clear from the present results if the decrease in AAV DNA synthesis with the Ad early region 2 mutants reflects a direct effect on AAV DNA synthesis or AAV protein synthesis. The experiments reported here (Fig. 3) do not clearly resolve AAV replicating form DNA synthesis from progeny strand synthesis. We have recently obtained evidence that in human cells the synthesis of AAV progeny strands, but not AAV replicating form DNA replication, is apparently dependent on AAV capsid protein synthesis or assembly or both (44; M. W. Myers and B.J. Carter, manuscript in preparation). Thus, the observed decrease in AAV DNA synthesis with the Ad mutants might be a secondary effect resulting from a decreased amount of AAV proteins. Alternatively, the 50- to 200-fold decrease in assembled AAV particles may result from a mass action effect of the decreased synthesis of both AAV DNA and AAV proteins.

As well as the decrease in AAV capsid proteins, the cytoplasmic concentrations of some AAV RNAs, especially the spliced RNAs, *c* and *g*, but apparently not RNA *a*, which contains a similar splice, were also diminished when Ad5*ts*125 was the helper at 40°C. The preferential decrease of some but not all cytoplasmic AAV RNA species cannot be directly accounted for by decreased AAV DNA synthesis as all the AAV RNAs are transcribed from overlapping regions of the genome. Our results suggest that the E72 protein may be involved in the positive modulation of certain AAV RNAs. We have previously noted (40) that the cytoplasmic AAV RNAs (Fig. 6) might be derived either by synthesis from several promoters or by posttranscriptional processing from a common precursor. Our present results may indicate that some function of the E72 protein affects the posttranscriptional processing of certain AAV RNAs. Analyses of nuclear AAV RNA transcripts will resolve this.

If the E72 protein is involved in the positive modulation of some AAV RNAs, this would represent an additional pleiotropic function for this protein. AAV RNA synthesis begins after DNA synthesis and concurrently with late Ad RNA synthesis (9). It is therefore interesting that the E72 protein might also have a role in the positive modulation of posttranscriptional processing of some late Ad mRNA's. This idea was also suggested recently by Klessig and Grodzicker (36), who reported that mutations mapping in the E72 protein coding region of the Ad2 and Ad5 genomes overcome the host restriction of growth of human Ad's in most monkey cells. The precise nature of this monkey cell host-range host-range restriction for human adenoviruses is not clearly understood. It apparently involves a decreased accumulation in the cytoplasm of some spliced, late Ad mRNA's, notably that for fiber protein (35, 36), and may also involve a defect in the translation of late Ad mRNA (1, 15, 18, 35, 45). Human AAV also exhibits a similar host restriction in monkey cells, and it was recently suggested that this restriction was due to a failure to translate AAV RNA (8), but in that study, individual AAV RNAs were not examined. Our present results do not exclude a role for the E72 protein in AAV RNA translation.

Whether the AAV helper functions affected by the Ad5*ts*125 or Ad5*ts*107 mutations reflect a direct involvement of the Ad E72 protein is not determined. AAV multiplication may require some other Ad protein dependent on functional E72. Any other such Ad protein would likely be an early protein as our results and those of others (51) with the Ad5*ts*149 mutant, as well as recent studies with microinjection of Ad mRNA into permissive cells (49), show that only Ad early genes are required for the expression of a complete AAV helper function. Alternatively, some effects of E72 may be mediated by interaction with host cell components, as has been inferred from the Ad host-range studies (36). For Ad, these properties can only be directly studied in the host-range system, as in the permissive human cell system they may be obscured by the early functions of E72. Thus, AAV may offer an alternative and independent probe to analyze certain functions of E72 in permissive human cells.

The studies described above collectively suggest additional similarities in the pleiotropic properties of the Ad E72 protein and simian virus 40 (SV40) T-antigen. SV40 T-antigen (reviewed in reference 41) also exerts negative control on SV40 early mRNA (48) and is required for SV40 DNA replication. Recently, SV40 T-antigen has been implicated in the positive control of SV40 late gene expression (33, 47). SV40 T-antigen can overcome the monkey cell host restriction for human Ad's (23, 30, 34) and AAV (8) and may also complement the Ad5*ts*125 lesion at the restrictive temperature in human cells (41).

Other workers (24, 51) reported that the Ad5*ts*125 mutant was an efficient helper of AAV. The reason for the difference between their observations and ours is not clear. Straus et al. (51)

did not assay AAV antigen synthesis, protein synthesis, or particle production, and their analysis would probably not have detected the type of differences in RNA species that we observed. They also used KB cell suspension cultures at 39.5°C and the yields of infectious AAV, even with an Ad5wt helper, were at best only of the order of 30 to 100 IU per cell. Similarly, Handa et al. (24) used HEK cells and obtained similar yields of AAV. These yields are about two logs lower than those we observed in our experiments with a wild-type or Ad5*ts*149 helper at 40°C. This last point may be important as the defect with Ad5*ts*125 for AAV growth does not appear to be an all-or-none phenomenon. Rather, the Ad5*ts*125 lesion appears to be affecting the efficiency of AAV growth. Whether this reflects the mechanism of the helper function or the innate degree of leakiness of the mutant is not clear. Other studies (28, 29, 42) have employed DNA-negative mutants of Ad31 or an avian Ad (CELO), but the mutations in these viruses have not been accurately mapped. Therefore, these results cannot readily be compared with ours.

Regardless of the precise nature of its involvement, our results with Ad5*ts*125 and Ad5*ts*107 suggest that the early region 2 of Ad is important for AAV growth. It is of interest that our results with Ad5*ts*149, which confirm those of others (24, 51), indicate that the Ad early region 5 protein, which is altered by this mutation and is required for Ad DNA synthesis, is not required for AAV growth. Recently, with Ad5 mutants having deletions in early region 1 (31), we have also shown that the expression of both complementation groups of this region (i.e., region 1a and 1b) are apparently required for AAV DNA replication and viral growth (Laughlin et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, S 160, p. 266). These studies collectively identify specifically mapped Ad genes which are apparently involved in some way in Ad helper functions for AAV. This is expected to aid in the biochemical analysis of these functions with respect to the gene expression of AAV, as well as that of Ad.

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