JOURNAL OF VIROLOGY, Jan. 1988, p. 206–210
0022-538X/88/010206-05$02.00/0

Vol. 62, No. 1

Adenovirus E1B 55-M_r Polypeptide Facilitates Timely Cytoplasmic Accumulation of Adeno-Associated Virus mRNAs

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Received 26 June 1987/Accepted 12 September 1987

Adenovirus provides helper functions that facilitate replication of adeno-associated virus (AAV). Both the adenovirus E1B 55-M_r and E4 34-M_r polypeptides are required for efficient and timely accumulation of AAV mRNA, proteins, and DNA. The E1B 55-M_r polypeptide is also required for rescue of the integrated AAV genome in Detroit 6-D5 cells in a normal time frame. All of these effects probably result from a single, primary delay in AAV mRNA accumulation. The AAV helper function provided by the E1B 55-M_r and E4 34-M_r polypeptides appears to closely parallel their normal role in the adenovirus replication cycle.

Although adeno-associated virus (AAV) can replicate under appropriate conditions in a helper-independent fashion (34), its replication is dramatically enhanced by coinfection with adenovirus (1), herpes simplex virus (4), or vaccinia virus (29). Five adenovirus genes have been implicated in helper function: E1A, E1B, E2A, E4, and VAI RNA (reviewed in references 3 and 5).

The adenovirus E1B region encodes two major products: a 21-M_r polypeptide and a 55-M_r moiety. These two polypeptides are coded in different, partially overlapping reading frames. The role of the adenovirus E1B gene products in helper function has not been clear. An adenovirus type 5 (Ad5) host range mutant, *hr*6 (11), with lesions in the region encoding the E1B 55-M_r polypeptide (*hr*6 contains both a 3-base-pair deletion at sequence positions 2346 through 2348 and a single-base-pair substitution at position 2947 [33]), served as a good helper for AAV DNA replication (15, 21) but failed to efficiently facilitate excision of the integrated AAV genome in Detroit 6 cells (18, 21). An Ad5 E1B deletion mutant, *dl*313 (deletion extends between sequence positions 1333 and 3640 [17, 30]), lacking both E1B 21- and 55-M_r polypeptide coding regions, completely failed to help AAV replication (19).

To better evaluate the role of adenovirus E1B gene products in the AAV replication cycle, we examined the ability of two Ad5 E1B deletion mutants to facilitate AAV growth. Mutant *dl*337 fails to produce the E1B 21-M_r polypeptide (22), whereas *dl*338 does not code the E1B 55-M_r moiety (23). *dl*337 was an efficient helper, whereas *dl*338 was not, clearly defining a role for the E1B 55-M_r polypeptide in AAV replication. Analysis of the *dl*338 helper defect indicated that the E1B product functions to enhance AAV mRNA accumulation posttranscriptionally. AAV DNA replication and rescue of the viral genome from the latent state were facilitated by *dl*338, but only after a substantial delay as compared with wild-type Ad5. Mutant *dl*355, which fails to produce the adenovirus E4 34-M_r polypeptide (10) that normally exists in a complex with the E1B 55-M_r polypeptide (8, 27), exhibits AAV helper defects similar to those of *dl*338.

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MATERIALS AND METHODS

Plasmids, viruses, and cells. The recombinant plasmid pSM620, in which the AAV-2 genome has been cloned into the *Pst*I site of pBR322 via poly(G) · poly(C) tails, has been described (25).

AAV-2 virus was obtained by transfection of 293 cells with pSM620 in the presence of an adenovirus helper, *dl*309. AAV-2 virions were purified from the resulting cell lysates by equilibrium density centrifugation. The parental Ad5 isolate is designated *wt*300 (16). *dl*309 is a phenotypically wild-type derivative of *wt*300 (17). *dl*337 (22) and *dl*338 (23) are derived from *dl*309 and contain deletions within the regions encoding the E1B 21- and 55-M_r polypeptides, respectively. *dl*337 lacks the sequence between positions 1770 and 1915, and *dl*338 lacks the sequence between positions 2805 and 3329. *dl*339 contains both 337 and 338 deletions (20). *dl*355 (10) lacks 14 base pairs within the E4 34-M_r coding region.

The 293 cell line is a human embryonic kidney cell line that contains and expresses the left 11% (regions E1A and E1B) of the Ad5 genome (9). HeLa cells were from the American Type Culture Collection (Rockville, Md.). Detroit 6-D5 cells are a subclone of Detroit 6 cells which harbor integrated, rescuable AAV genomes (6).

RNA preparation and analysis. The protocols for isolation of cytoplasmic RNA from infected HeLa cells and RNA blot analysis have been described previously (12). Double-stranded AAV DNA was labeled with ^{32}P-deoxynucleoside triphosphates by nick translation (24) and used as a probe.

Transcription rates were measured in isolated nuclei as described previously (23). The M13 Ad5 E2A probe carried the viral DNA segment from 63.6 to 68 map units, the actin probe was a chicken sequence from pA1 (7), and the AAV probe included the entire AAV chromosome (p*sub*201) (26).

Protein analysis. For analysis of AAV- or adenovirus-specific polypeptides, cultures were labeled with [^{35}S]methionine (100 mCi/ml; 1,100 Ci/mmol) for 30 min at various times after infection and then subjected to immunoprecipitation with a monoclonal antibody prepared against either purified AAV capsid proteins (L.-S. Chang, S. Pan, R. J. Samulski, and T. Shenk, manuscript in preparation) or the adenovirus E1B 55-M_r polypeptide (27). Immunoprecipitations and electrophoresis were as described previously (23).

DNA replication and rescue assays. HeLa cells were em-

ployed for replication assays, and Detroit 6-D5 cells that harbor integrated AAV genomes (6) were used for rescue assays. In both cases, low-molecular-weight DNA was prepared (14) at various times after infection and analyzed by DNA blot analysis (31) with a ^{32}P-labeled probe prepared by nick translation of double-stranded AAV DNA.

RESULTS

E1B 55-M_r polypeptide required for efficient AAV growth. We have previously described the construction and analysis of several deletion mutations that destroy either one or both of the major Ad5 E1B coding regions. *dl*337 (22) lacks the sequence between positions 1770 and 1915 and produces no 21-M_r polypeptide, *dl*338 (23) lacks the sequence between positions 2805 and 3329 and fails to synthesize the 55-M_r species, and *dl*339 (20) carries both deletions. To assay the ability of these mutant viruses to provide helper function to AAV, HeLa cells were transfected with pSM620, an infectious AAV-containing plasmid (25), in the presence of infecting wild-type or mutant adenoviruses. *dl*337 (E1B 21-M_r polypeptide negative) was as effective a helper as wild-type Ad5, whereas *dl*338 (E1B 55-M_r polypeptide negative) or *dl*339 (E1B 21-M_r and 55-M_r polypeptide negative) led to a 100-fold reduction in the yield of AAV virions at 40 h after coinfection (Table 1). Since the E1B55-M_r and E4 34-M_r polypeptides are known to exist in a complex (8, 27) and viruses with lesions in either gene display similar phenotypes (10), *dl*355 (lacks 14 bp within the E4 34-M_r polypeptide-coding region, [10]) was tested for its helper ability. No AAV yield was detected at 40 h after coinfection with this virus (Table 1).

We conclude that both the E1B 55-M_r and E4 34-M_r polypeptides provide helper function to AAV in HeLa cells, whereas the E1B 21-M_r moiety does not.

Cytoplasmic accumulation of AAV mRNA dependence on the E1B 55-M_r and E4 34-M_r polypeptides. To localize the defect in the AAV growth cycle that resulted from a lack of the E1B 55-M_r polypeptide, AAV RNA metabolism was analyzed. First, transcription rates were monitored. HeLa cell cultures were coinfected with AAV and either *dl*309 (wild type) or *dl*338 (E1B 55-M_r polypeptide negative); at 7 h after coinfection, nuclei were prepared, and transcription rates were assayed (Table 2). The rate of AAV transcription was reduced by approximately one-third in the absence of the E1B product. Next, steady-state levels of cytoplasmic, AAV-specific RNAs were assayed. Total cytoplasmic RNA was prepared at various times after coinfection of HeLa cells, applied to nitrocellulose with a slot-blot apparatus, and quantitated by hybridization with excess ^{32}P-labeled AAV probe DNA (Fig. 1). Substantial reductions in AAV RNA levels were observed in the mutant compared with the wild-type helper coinfections after 24 h. AAV RNA levels were 85-fold and 43-fold reduced for the *dl*338 (E1B 55-M_r polypeptide negative) and *dl*335 (E4 34-M_r polypeptide negative) coinfections, respectively. After 48 h, AAV mRNA levels in the mutant helper coinfections had caught up to the level observed for the wild-type helper.

These results argue that the E1B 55-M_r polypeptide functions posttranscriptionally to enhance cytoplasmic levels of AAV RNA. The E4 34-M_r polypeptide is also required for timely cytoplasmic accumulation of AAV RNA; even though transcription rates were not measured for this mutant, it also likely functions at a posttranscriptional level (see below). Cytoplasmic RNAs ultimately accumulate to near-normal levels late after coinfection with mutant helper viruses.

AAV capsid protein synthesis and DNA replication delayed in the absence of E1B 55-M_r or E4 34-M_r polypeptide. Synthesis of AAV capsid polypeptides was monitored by labeling coinfected HeLa cells with [^{32}S]methionine and subjecting whole cell extracts to immunoprecipitation with a monoclonal antibody produced by immunization with AAV virions (Fig. 2). Consistent with mRNA accumulation, capsid synthesis was delayed when either *dl*338 or *dl*355 served as the helper.

To assay the ability of mutant adenoviruses to provide help for AAV DNA replication, low-molecular-weight DNA was isolated at various times after coinfection of HeLa cells and analyzed by DNA blot analysis (Fig. 3). Both *dl*338 and

TABLE 1. Effect of adenovirus mutations on yield of AAV virions[a]

Helper	Deletion location	AAV yield (A_{260}/ml)
*wt*300	Wild-type Ad5	9.7
*dl*309	Wild-type parent	10.6
*dl*337	E1B 21-*M*, polypeptide	10.1
*dl*338	E1B 55-*M*, polypeptide	0.07
*dl*339	E1B 21-*M*, polypeptide and E1B 55*M*, polypeptide	0.09
*dl*355	E4 34-*M*, polypeptide	<0.02

[a] HeLa cells were transfected with pSM620 DNA in the presence of the indicated adenovirus at a multiplicity of 10 PFU/ml. At 40 h after infection, 2 × 10^7 cells were disrupted by sonication in buffered saline, debris was pelleted by low-speed centrifugation, and the virions in supernatants subjected to equilibrium centrifugation in CsCl. After centrifugation, bands of AAV virions were removed from the gradients, virions were precipitated with ethanol, and AAV DNA was prepared by pronase treatment and phenol extraction. DNA was suspended in 0.5 ml of buffer, and its concentration was determined.

TABLE 2. Transcription rates in cells coinfected with AAV and either wild-type Ad5 or *dl*338[a]

Coinfecting virus	^{32}P cpm hybridized		
	Actin	Ad5 E2A	AAV
None	100	10	40
*dl*309	100	220	1,390
*dl*338	100	245	890

[a] Nuclei were prepared from HeLa cells at 7 h after coinfection and incubated for 15 min at 30°C in the presence of [^{32}P]UTP, and nuclear RNA was prepared. RNA was hybridized to single-stranded probe DNA carried in M13 immobilized on nitrocellulose filters, and radioactivity was quantitated. Background radioactivity (approximately 20 cpm) that hybridized to M13-containing control filters has been subtracted from the results, which were then normalized to the actin probe.

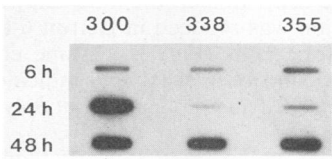

FIG. 1. Slot-blot analysis of AAV-specific cytoplasmic RNAs that have accumulated at various times after coinfection of HeLa cells with AAV and helper adenoviruses. Helper viruses (*wt*300, *dl*338, or *dl*355) were used at a multiplicity of 10 PFU per cell. Total cytoplasmic RNAs were isolated at the indicated times, applied to a nitrocellulose filter, and probed with excess ^{32}P-labeled AAV DNA.

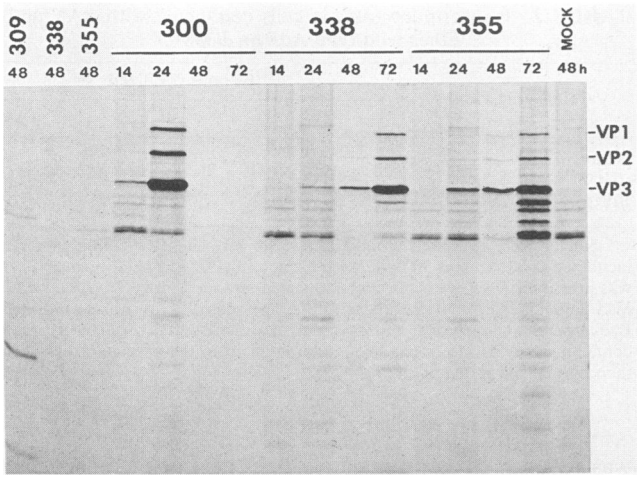

FIG. 2. Electrophoretic analysis of AAV capsid proteins synthesized at various times after coinfection of HeLa cells with AAV and helper adenoviruses. Helper viruses (*wt*300, *dl*309, *dl*338, or *dl*355) were used at a multiplicity of 10 PFU per cell. Cells were labeled with [^{35}S]methionine for 30 min at the indicated times, and extracts were prepared, subjected to immunoprecipitation with a monoclonal antibody prepared against AAV capsids, and analyzed by electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate. Bands corresponding to VP1, VP2, and VP3 are designated. The first three lanes at the left of the figure are control infections which received only helper viruses.

*dl*355 were able to facilitate AAV DNA replication, but only after a delay of many hours in comparison to wild-type Ad5. Half-maximal levels of AAV DNA accumulated by about 24 h with the wild-type helper, but not until about 72 h for *dl*338 (E4 34-M_r polypeptide negative) and 84 h for *dl*355 (E1B 55-M_r polypeptide negative).

We conclude that both AAV capsid protein biosynthesis and DNA replication are severely delayed in the absence of either the E1B 55-M_r or E4 34-M_r polypeptide. These delays are presumably, at least in part, direct consequences of the defect in AAV mRNA accumulation.

Excision and replication of AAV DNA within Detroit 6-D5 cells delayed in the absence of the E1B 55-M_r polypeptide. To probe for a role of the adenovirus E1B gene product in excision and replication of a latent AAV genome, Detroit 6-D5 cells, which contain integrated AAV sequences (6), were infected with either *dl*309 or *dl*338. Low-molecular-weight DNA was prepared 24 h later and assayed for the presence of free-replicating AAV DNA by blot analysis (Fig. 4). AAV DNA was detected only in the cells infected with wild-type adenovirus. As a control, AAV virus was included with the adenovirus inoculum, and AAV DNA replication was evident after 24 h in the presence of either helper. Thus, AAV replication requirements can be provided by the *dl*338 helper in these cells. In fact, the time delay for *dl*338-helped AAV replication was reduced in Detroit 6-D5 cells as compared with HeLa cells (Fig. 3). A time course for *dl*338-mediated rescue in Detroit 6-D5 cells indicated that the AAV genome was excised and replicated, but only after a substantial delay (Fig. 4).

The E1B 55-M_r polypeptide is clearly required for efficient rescue and replication of the integrated AAV chromosome. It is not possible to conclude whether the excision step per se is defective. If, indeed, excision is an event independent of replication, it could occur at normal efficiency after infection with *dl*338. Since the excised sequences would undergo a substantial lag before replication, they would not be detected in our assay.

Ad5 E1B 55-M_r protein–E4 34-M_r protein complex not detectably altered by AAV. As mentioned above, the Ad5 E1B 55-M_r and E4 34-M_r polypeptides exist in a complex (8, 27). The complex can be detected by coprecipitation of both polypeptides with a monoclonal antibody specific for the E1B 55-M_r polypeptide. Since the polypeptides comprising the complex play a key role in AAV gene expression, it was of interest to determine whether the complex was detectably altered in the presence of AAV. Accordingly, cultures were labeled with [^{35}S]methionine for 30 min at various times after HeLa cells were either infected with wild-type adenovirus (*dl*309) alone or coinfected with *dl*309 plus AAV. Whole cell extracts were prepared and subjected to immunoprecipitation by using a monoclonal antibody specific for the E1B 55-M_r polypeptide, 2A6 (28). AAV induced no detectable difference in the time of appearance of the complex or in its constituents (Fig. 5).

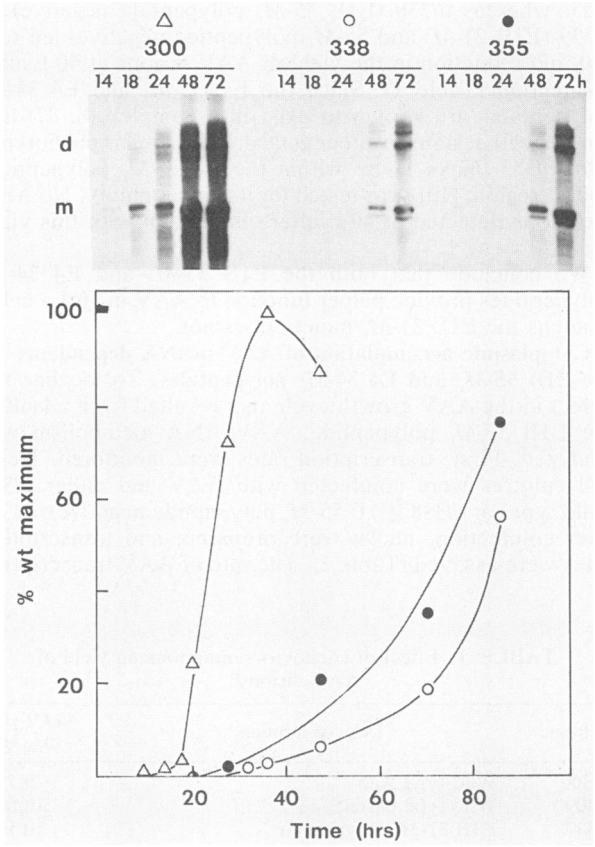

FIG. 3. Analysis of AAV DNA accumulation at various times after coinfection of HeLa cells with AAV and helper adenoviruses. Helper viruses (*wt*300, *dl*338, *dl*355) were used at a multiplicity of 10 PFU per cell. Low-molecular-weight DNA was prepared at the indicated times and subjected to DNA blot analysis with ^{32}P-labeled AAV DNA as a probe. The top of the figure displays the autoradiogram obtained from such an experiment. Monomer (m) and dimer (d) AAV DNA molecules are labeled. The bottom of the figure displays a second experiment in which bands corresponding to AAV monomers and dimers were cut from the nitrocellulose sheet. Their radioactivity was quantitated and then plotted as a function of time. Symbols: △, *wt*300; ○, *dl*338; ●, *dl*355.

DISCUSSION

The E1B 55-M_r and E4 34-M_r polypeptide but not the E1B 21-M_r polypeptide is required for efficient adenovirus helper function (Table 1). In the absence of either the E1B 55-M_r or E4 34-M_r moiety, delays occurred in the accumulation of AAV-specific cytoplasmic RNA (Fig. 1), capsid proteins (Fig. 2), and DNA replication (Fig. 3). The pattern of delays suggests that the primary helper function of the E1B and E4 gene products is to facilitate cytoplasmic accumulation of AAV mRNAs, since a delay in AAV translation would naturally follow and DNA replication is known to be dependent on AAV gene products (13, 32).

Since AAV transcription rates are nearly normal in the absence of the E1B 55-M_r polypeptide (Table 2), the adenovirus product must function posttranscriptionally to facilitate AAV mRNA accumulation. This fits well with the protein's function in the adenovirus replication cycle. The E1B-negative mutant *dl*338 displays normal transcription rates for late mRNAs but reduced rates of cytoplasmic RNA accumulation (23). Since this mutant exhibits normal nuclear processing and cytoplasmic mRNA half-lives, we have proposed that the function of the E1B polypeptide is to facilitate transport of late viral mRNAs from the nucleus to the cytoplasm. A similar phenotype was observed for *dl*355 (E4 34-M_r polypeptide negative) (10). Other groups have studied different mutants with mutations in the E1B 55-M_r polypeptide and reached similar conclusions (2, 33). It seems reasonable to suggest that the E1B 55-M_r polypeptide–E4 34-M_r polypeptide complex performs exactly the same function for AAV mRNAs as for adenovirus mRNAs. It is interesting to note that the complex facilitates accumulation of late but not early adenovirus mRNAs (23). Thus, AAV mRNAs are treated like late adenovirus mRNAs in coinfected cells.

It is easy to rationalize earlier studies on the role of E1B products in AAV replication given our present results.

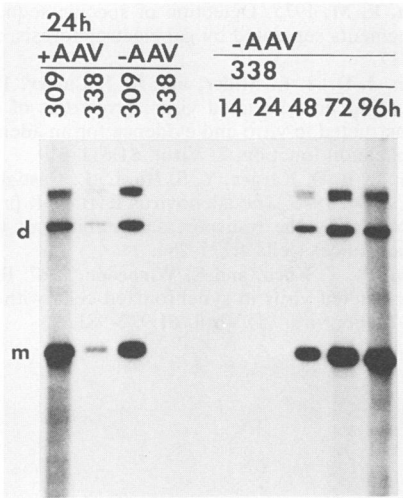

FIG. 4. Analysis of AAV DNA rescued from Detroit 6-D5 cells after infection with either wild-type or mutant adenovirus. Cells were infected with either *dl*309 or *dl*338 at a multiplicity of 10 PFU per cell, and low-molecular-weight DNA was isolated and analyzed by DNA blot analysis. The two leftmost lanes display controls in which cells were coinfected with adenovirus and AAV, and the next two lanes display AAV DNAs detected 24 h after infection with *dl*309 or *dl*338 alone. The right portion of the figure displays a time course of AAV rescue after infection with *dl*338. Monomer (m) and dimer (d) AAV DNA molecules are labeled.

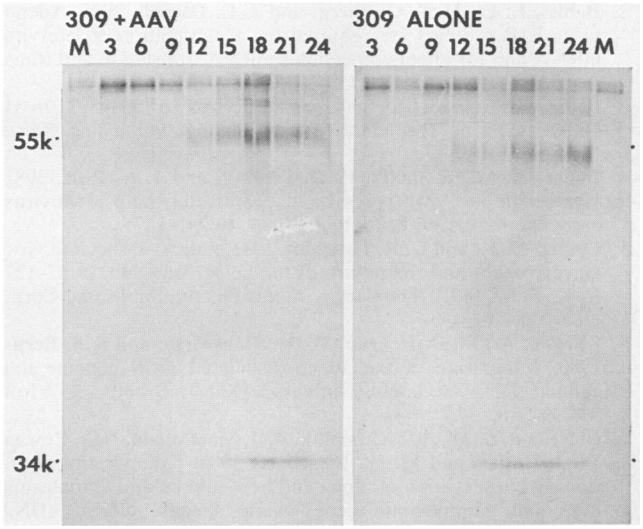

FIG. 5. Electrophoretic analysis of the E1B 55-M_r polypeptide–E4 34-M_r polypeptide complex formed in adenovirus-infected HeLa cells in the presence or absence of AAV. Cells were infected with both *dl*309 and AAV or *dl*309 alone. At the indicated times after infection, cells were labeled for 30 min with [^{35}S]methionine, and extracts were prepared, subjected to immunoprecipitation with a monoclonal antibody specific for the E1B 55-M_r polypeptide, and analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. The positions of bands corresponding to E1B 55-M_r and E4 34-M_r polypeptides are designated.

Ostrove and Berns (21) assayed AAV DNA accumulation at 48 h after coinfection with strain *hr*6 (E1B 55-M_r polypeptide negative) and found that AAV had replicated. Laughlin et al. (19) assayed much earlier, at 20 h after coinfection with *dl*313 (E1B negative), and found no evidence for AAV replication. The marked delay in accumulation of AAV DNA after coinfection with the E1B 55-M_r polyprotein-negative mutant *dl*338 (Fig. 3) suggests the key variable in earlier experiments was the time of assay. It is possible, however, that part of the *dl*313 helper defect results from alteration of the 3′ exon of E1A mRNAs by its deletion (17).

Finally, adenovirus E1B 55-M_r polypeptide function is required for timely rescue of the AAV genome from the integrated state in Detroit 6-D5 cells (Fig. 4). Rescue involves both excision and replication of the AAV genome. It is not clear whether these are separable events; excision may be a replicative process. The E1B 55-M_r polypeptide may not function directly in either process. Again, the delay in rescue could result entirely from a delay in the accumulation of AAV mRNAs whose polypeptide products are required for the rescue process.

ACKNOWLEDGMENTS

We thank L.-S. Chang and S. Pan for the gift of monoclonal antibody prepared against AAV capsid proteins.

This work was supported by Public Health Service grant CA 39606 from the National Cancer Institute. R. J. Samulski was supported by Public Health Service postdoctoral fellowship CA 07295 from the National Cancer Institute, and T. Shenk is an American Cancer Society Research Professor.

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