Adenovirus L1 52- and 55-Kilodalton Proteins Are Required for Assembly of Virions

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A variant of adenovirus type 5 that contained a mutation within the L1 52- and 55-kilodalton (52/55K) protein-coding region was isolated. The mutant, termed *ts369*, produced L1 52/55K proteins with a two-amino-acid substitution and was temperature sensitive. Temperature-shift experiments indicated that the *ts369* defect was late in the viral growth cycle. DNA replication and synthesis of late proteins occurred normally in *ts369*-infected cells at the nonpermissive temperature, but mature virions were not produced. Rather, capsidlike particles associated with the left-terminal region of the viral chromosome accumulated. These incomplete particles could not be chased into mature virions when the infected cells were shifted to the permissive temperature. However, previously synthesized proteins could be assembled into virions in the presence of a protein synthesis inhibitor upon shutdown from the nonpermissive temperature, suggesting that the inactivation of the L1 52/55K proteins was reversible. These results indicate that the adenovirus L1 52/55K proteins play a role in the assembly of infectious virus particles.

The adenovirus major late transcription unit encodes a primary transcript that is about 29,000 nucleotides in length (13, 27). This transcript is processed by differential poly(A) site utilization and splicing to generate at least 18 distinct mRNAs. These mRNAs have been grouped into five families, termed L1 to L5, on the basis of utilization of common poly(A) addition sites (6, 27, 42).

Although the major late promoter is active early after infection, only the 5'-proximal L1 family of mRNAs accumulates in the cytoplasm at this time (1, 5, 24, 28, 32, 34). This finding results from transcriptional termination midway through the transcription unit and preferential utilization of the L1 poly(A) addition site (5, 21, 24, 28). The termination is partially relieved as the infection enters the late phase, allowing expression of the full set of mRNAs encoded by the major late unit.

The L1 family of mRNAs encodes three known proteins: the 52- and 55-kilodalton (52/55K) proteins and the IIIa protein (Fig. 1A). IIIa is a structural component of the virion. The 52/55K proteins have not been detected in virions (25, 26). These two proteins are highly related in amino acid sequence, are phosphorylated, and are localized primarily near the inner surface of the nuclear membrane (26). Although mRNAs encoding the 52/55K protein are present in the cytoplasm early after infection, their protein products are not detected until 6 to 7 h after infection (26).

A variety of temperature-sensitive mutations have been mapped to the L1 region (12, 31, 39, 41), but it is not clear that any of the lesions alter the 52/55K-coding region.

To investigate the function of the 52/55K proteins, we have generated a mutant virus, *ts369*. This variant contains a mutation within the L1 coding region that produces a two-amino-acid substitution within the L1 52/55K proteins. It is temperature sensitive for lytic growth. Analysis of the physiological defect displayed by *ts369* at the nonpermissive

temperature indicates that the 52/55K proteins play a role in assembly of virions.

MATERIALS AND METHODS

Cells and viruses. *d1308/310* is a phenotypically wild-type adenovirus type 5 (Ad5) variant that carries a series of altered restriction endonuclease cleavage sites (22). The 293 cell line is a human embryonic kidney line transformed with a DNA fragment comprising the left 11% of the Ad5 genome (16). HeLa and 293 cells were propagated in monolayer cultures in medium supplemented with 7 to 10% calf serum. For electron microscopic analysis, HeLa cells were propagated as spinner cultures in medium supplemented with 5% calf serum. Plaque-purified stocks of *d1308/310* and *ts369* were prepared as lysates of infected 293 cells grown at 32°C. These lysates were frozen and thawed four times and then titrated by plaque assay on 293 cells at 32°C. Cells were infected at a multiplicity of 20 PFU/ml unless otherwise noted.

Construction of the 52/55K mutant. Plasmid pA5-SalC (36), which contains Ad5 sequences from 29 to 45 map units, was used to generate site-specific mutations. pA5-SalC was cut with *EcoRI*, and the resulting ends were filled in with the Klenow fragment of DNA polymerase I and religated to generate a plasmid lacking an *EcoRI* site, termed pSalC-R1⁻. pSalC-R1⁻ was cleaved at the *SacI* site within the 52/55K-coding region and T4 nuclease treated to remove the single-stranded ends; then an 8-base-pair *EcoRI* linker with the sequence GGAATTCC was inserted. This procedure generated a +1 frameshift in the 52/55K open reading frame. The plasmid was then cut with *EcoRI*, digested with mung bean nuclease to remove the single-stranded ends, and religated. This treatment removed 4 base pairs, recovered the open reading frame, and generated an *ApaI* site. These manipulations generated a two-amino-acid change (Fig. 1). This new plasmid was called pSac-Apa⁺.

The 29- to 45-map-unit portion from pSac-Apa⁺ was cloned into a larger plasmid containing 0 to 45 map units, p45WT. The 0- to 45-map-unit portion, which now contained

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FIG. 1. Diagram of the L1 coding region and sequence alteration in *ts369*. (A) mRNAs that give rise to the 52/55K and IIIa proteins. Symbols: →, mRNAs; /, introns; □, protein-coding regions. Positions of the mRNAs in map units are indicated at the bottom, as is the location of the mutation in *ts369*. Exons comprising the three elements of the tripartite leader sequence and i-leader are designated 1, 2, 3, and (i), respectively. (B) Sequence of wild-type and mutant viral DNA and protein at the position of the *ts369* mutation. Sequence positions for Ad2 (15) are indicated above the nucleotide sequence, and the amino acid sequence is listed below. The change in *ts369* is marked by a bracket, and restriction endonuclease cleavage sites that discriminate wild-type from mutant DNAs are listed above the brackets.

the mutation, was reconstructed into intact virus by in vivo recombination with a 35- to 100-map-unit fragment essentially as described by Chinnadurai et al. (4). The two purified fragments were cotransfected into semiconfluent 293 cells by using a variation of the calcium phosphate technique described by Parker and Stark (29). Four hours after transfection, the cells were treated for 1 min with 20% glycerol in Tris-buffered saline, which increased the efficiency of transfection approximately 10-fold. Cultures were overlaid with medium containing agar and 4% calf serum. Plaques were visualized by staining with neutral red and isolated after 10 days at 32°C. Virus stocks were prepared from several plaques, and the presence of the mutation was checked by *Apa*I digestion of purified viral DNA. Stocks used for further experiments were plaque purified three times, and the 0- to 15-map-unit region was marker rescued to remove fortuitous mutations that arose during the passaging of plasmids in bacteria. The resultant mutant was termed *ts369*.

Analysis of viral proteins. To label infected 293 cells for analysis of viral proteins, the medium was removed and replaced with methionine-deficient (2.5% the normal concentration) medium supplemented with ³⁵S Translabel (50 μCi/ml; 1,000 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) and 2% calf serum. After 1 h, cells were washed in ice-cold phosphate-buffered saline (PBS) and recovered in 0.25 ml of TD buffer (25 mM Tris, 5 mM KCl, 138 mM NaCl, 0.37 mM Na₂HPO₄, pH 7.5) per 10⁷ cells. An equal volume of 2× electrophoresis sample buffer (10) was added, and the sample was frozen at -20°C.

To analyze labeled proteins, samples were boiled for 5 min before being loaded onto a 12.5% polyacrylamide gel prepared by the method of Dreyfuss et al. (10). After electrophoresis, the gels were fluorographed with Resolution (EM Corp., Chestnut Hill, Mass.), dried under vacuum, and exposed to Kodak X-Omat AR or RP film (Eastman Kodak Co., Rochester, N.Y.).

Analysis of virions and assembly intermediates. HeLa monolayer cultures (2 × 10⁶ to 4 × 10⁶ cells per 100-mm-diameter dish) were infected at 100 PFU per cell. At 21

h postinfection, the medium was removed and cells were washed with warm PBS. The infected cells were then labeled with 10 μCi of ³⁵S Translabel per ml in methionine-deficient medium supplemented with 5% calf serum (dialyzed against PBS to remove methionine). Alternatively, the infected cells were labeled with 10 μCi of [³H]thymidine per ml (45 Ci/mmol) in medium supplemented with 5% calf serum. The infected, labeled cells were harvested at 48 h postinfection, washed with ice-cold PBS, and frozen in 1 ml of TD buffer per 10⁷ cells.

Infected cells were pulse-labeled for temperature shift experiments. HeLa cells were infected at 100 PFU per cell as described above. The cells were washed at 26 h postinfection with warm PBS and then labeled with 1 ml of ³⁵S Translabel (200 μCi/ml) in methionine-deficient medium supplemented with 5% dialyzed calf serum. After 1 h, cells were washed with warm PBS, and medium supplemented with 50 times the normal concentration of unlabeled methionine (1.5 mg/ml) was added. After 3 h, cells were harvested or chased by removal of medium and readdition of fresh medium containing an excess of methionine, warmed to the required temperature. In some experiments, protein synthesis was blocked by the addition of cycloheximide (20 μg/ml) to the methionine-supplemented medium during the chase period.

To analyze radiolabeled assembly intermediates, cell pellets were frozen and thawed four times, and then debris was removed by centrifugation at 2,600 × g. The supernatant from approximately 10⁷ cells was layered onto a preformed linear CsCl density gradient (1.2 to 1.4 g/cm³) and centrifuged at 62,500 × g for 3 h at 10°C. After centrifugation, 0.4-ml fractions were pumped from the bottom of the tube, a portion of each fraction was mixed with liquid scintillant, and the radioactivity measured in a scintillation counter.

For analysis of proteins in virions and assembly intermediates, samples were concentrated by ethanol precipitation. To each sample, an equal volume of TNE (10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 7.5) was added, followed by 2 volumes of ethanol. The samples were allowed to precipitate overnight at -70°C. The samples were spun at top speed in

a microfuge at 4°C for 30 min, washed with 70% ethanol, and dried. The pellet was recovered in 12 µl of 2× electrophoresis sample buffer. Electrophoresis and autoradiography were performed as described above.

To analyze the DNA in virions or incomplete particles, samples were purified by two rounds of CsCl density gradient centrifugation, ethanol precipitated as described above, and then recovered in TNE containing 0.5% sodium dodecyl sulfate, 0.013 mM EDTA, and proteinase K (final concentration, 0.36 mg/ml). In the case of incomplete particles, 1 µg tRNA was added as a carrier. The mix was incubated at 37°C for 1 h and then extracted twice with TNE-saturated redistilled phenol, once with equal volumes of phenol and chloroform-isoamyl alcohol (24:1, vol/vol), and once with chloroform-isoamyl alcohol. The aqueous phase was ethanol precipitated, recovered in TNE, and extracted once again with phenol as described above. The samples were precipitated with ethanol and suspended in TE (10 mM Tris, 1 mM EDTA, pH 8).

DNA was analyzed by restriction digestion, followed by DNA blot analysis using as ³²P-labeled probe DNA the entire *d/308/310* genome.

Electron microscopy. Spinner cultures of HeLa cells were grown to a concentration of 5×10^5 cells per ml at 37°C. Cells were spun at $600 \times g$, and the pellet of cells was suspended in 1/10 the original volume of medium supplemented with 0.5% calf serum and virus to 20 PFU per cell. The cells were incubated for 1 h at 39.5°C, after which time fresh warmed medium was added. At 36 h after infection, 50 ml of the culture was removed, and cells were pelleted by centrifugation at $600 \times g$ for 3 min at room temperature. The cells were washed twice with room temperature medium and pelleted at $600 \times g$ in an Ultra-Clear centrifuge tube (9/16 by 3.5 inches [ca. 1.4 by 8.9 cm]; Beckman Instruments, Inc., Fullerton, Calif.). The cell pellet was fixed for 15 min at room temperature in 2.5% glutaraldehyde in serum-free medium. The pellet was washed three times in ice-cold 0.1 M sodium cacodylate, pH 7.2, and then briefly postfixed with 1% OsO₄ in 0.1 M sodium cacodylate for 3 min at 4°C. The cells were subsequently washed in 0.1 M sodium cacodylate and then dehydrated through a series of increasing ethanol concentrations, ending with three changes of 100% ethanol. The sample was transferred through a graded series of propylene oxide-resin mixtures (1:2, 1:1, and 2:1) to epon-araldite. After infiltration with three changes of the resin, the samples were transferred to fresh resin and allowed to polymerize for 48 h at 60°C. Thin sections were cut and poststained with 10% uranyl acetate and lead citrate before visualization.

RESULTS

Isolation and growth properties of *ts369*. The adenovirus L1 transcription unit contains two major open reading frames, which encode the 52/55K proteins and the IIIa structural protein (Fig. 1A). A series of small, in-frame insertion and substitution mutations was introduced into the 52/55K-coding region within a plasmid. The mutant sequences were then used in attempts to recover intact viable viruses by overlap recombination (4) of chromosomal DNA segments. Only one of the mutant sequences generated a virus that could be propagated. This virus, termed *ts369*, contains a two-amino-acid substitution (Glu-Leu to Gly-Pro; Fig. 1B) within the 52/55K-coding region.

The ability of *ts369* to form plaques was found to be temperature sensitive. When the *ts369* yield was titrated at

TABLE 1. Comparison of yields of mutant and wild-type viruses propagated at 32 versus 39.5°C^a

Virus	Yield (PFU/ml)					
	48 h		72 h		96 h	
	32°C	39.5°C	32°C	39.5°C	32°C	39.5°C
<i>ts369</i>	6×10^7	4×10^4	3×10^8	3×10^5	3×10^7	3×10^4
<i>d/308/310</i>	8×10^7	3×10^8	3×10^8	5×10^7	10^7	10^7

^a Human 293 cells were infected at a multiplicity of 20 PFU per cell; cultures were maintained at 32 or 39.5°C and harvested at the indicated times. Yields were determined by plaque assay on 293 cells at 32°C.

32 versus 39.5°C, the permissive and nonpermissive temperatures, respectively, it produced 10⁵ fewer plaques at the nonpermissive temperature. Furthermore, the yield of *ts369* was reduced by a factor of 10³ when it was propagated at 39.5°C as compared with 32°C (Table 1). The yield obtained by growth of *ts369* at 39.5°C consisted primarily of wild-type revertants (data not shown), explaining the greater difference in yield observed by direct plaque assay of a mutant stock (10³-fold) as compared with growth at the two temperatures (10³-fold). The high-temperature block was not leaky; extended incubation of a *ts369* plaque assay at 39.5°C did not eventually lead to enhanced yields. The mutation in *ts369* proved to be recessive; it was complemented in *trans* by wild-type virus (data not shown).

The temperature-sensitive phenotype of *ts369* is due to the two-amino-acid substitution within the L1 52/55K protein. Three temperature-independent revertants of *ts369* were isolated, and each contained a new alteration at the site of the original mutation (data not shown).

***ts369* exhibits a defect late in the viral growth cycle.** The critical time for expression of the 52/55K proteins was determined by performing a temperature shift experiment. Human 293 cells were infected with *ts369* or *d/308/310*, a phenotypically wild-type Ad5 variant, at 32 or 39.5°C and then shifted to the alternate temperature at various times postinfection. The infected cultures were all harvested at 72 h postinfection, and infectious virus was titrated by plaque assay at 32°C to determine the effects of incubation at the nonpermissive temperature on virus yield. Figure 2 compares the yield of *ts369* with that obtained in a *d/308/310* infection treated in the same manner. *ts369* appeared to be blocked late in infection. Treatment for up to 12 h at the nonpermissive temperature had little effect on the final yield. The late block led to a 10⁴-fold reduction in *ts369* virus yield at the nonpermissive temperature.

To determine whether the block in virus yield was due to a defect in DNA replication, DNA blot analysis was performed, using an adenovirus-specific probe and DNA isolated from *ts369*-infected cells incubated for various time periods at 32 or 39.5°C. DNA replication was normal at the nonpermissive temperature (data not shown).

To ascertain whether late protein synthesis was disrupted in a *ts369* infection at the nonpermissive temperature, 293 cells were labeled with a mixture of [³⁵S]methionine and [³⁵S]cysteine for 1 h at 12, 24, or 36 h after infection with either *ts369* or *d/308/310*. Cells were harvested immediately after labeling, and proteins were analyzed by electrophoresis. Late protein synthesis occurred at wild-type levels in a *ts369* infection at the nonpermissive temperature (Fig. 3). Shut-off of host cell protein synthesis occurred as well, as is evident from the shift in protein labeling pattern between 12 and 24 h postinfection for both *ts369*- and *d/308/310*-infected cells.

FIG. 2. Temperature shift experiment performed with *ts369* and its wild-type parent, *dl308/310*. Human 293 cells were infected with either mutant or wild-type virus at a multiplicity of 20 PFU per cell. Infections were performed either at 39.5°C and shifted to 32°C at the indicated times (○) or at 32°C with a shift to 39.5°C at the indicated times (△). All infected cultures were harvested at 72 h after infection, and virus yields were determined by plaque assay on 293 cells at 32°C.

FIG. 3. Electrophoretic analysis of polypeptides synthesized in 293 cells at various times after infection with *ts369* or its wild-type parent. Cells were infected at a multiplicity of 20 PFU per cell and labeled for 1 h with a mixture of [³⁵S]methionine and [³⁵S]cysteine at the indicated times (hours) postinfection (hpi). An equal volume of each lysate was analyzed by electrophoresis. Radioactive marker polypeptides (molecular weights designated on the left) and mock-infected cells were included as controls.

FIG. 4. Density gradient analysis of particles assembled in HeLa cells after infection with *ts369* or its wild-type parent. Cells were infected at a multiplicity of 100 PFU per cell with either *dl308/310* (A) or *ts369* (B) and labeled with a mixture of [³⁵S]methionine and [³⁵S]cysteine or with [³H]thymidine from 21 to 45 h after infection. Cells were then harvested, and particles were extracted and subjected to centrifugation on preformed 1.2- to 1.4-g/cm³ CsCl gradients. Gradients were fractionated, and radioactivity was quantitated in a scintillation counter (○, ³⁵S; ●, ³H). The densities of fractions (△) were monitored by measuring refractive index.

Accumulation of partially assembled virus particles in *ts369*-infected cells. Since *ts369* infection produced both DNA and late proteins, it was of interest to see whether virus particles were assembled at the nonpermissive temperature. HeLa cells were infected at 100 PFU per cell with *dl308/310* or *ts369*, labeled from 21 to 45 h postinfection with a mixture of [³⁵S]methionine and [³⁵S]cysteine or with [³H]thymidine, and then harvested. The particles were extracted and analyzed on a preformed 1.2- to 1.4-g/cm³ linear CsCl gradient. In a wild-type virus infection, a major peak with a density of 1.34 g/cm³ was observed (Fig. 4A). Both on the basis of its density and because it was labeled with precursors to both DNA and protein, this peak was judged to be composed of virions. Also evident was a broad peak of ³⁵S-labeled material with a median density of about 1.29 g/cm³. These structures were not significantly labeled with [³H]thymidine, implying that a negligible amount of DNA, if any, was present within these particles. *ts369* did not accumulate mature virions at the nonpermissive temperature (Fig. 4B). Rather, a particle with a density of 1.30 to 1.31 g/cm³ was observed. Direct visual inspection of a CsCl gradient containing *ts369* particles revealed a doublet of bands at the position of the 1.30- to 1.31-g/cm³ peak. Unfortunately, these two populations were not separated in the gradient fractions. These structures appeared to lack appreciable amounts of viral DNA, as seen by the lack of [³H]thymidine incorporation. Electron microscopic analysis demonstrated that the 1.30- to 1.31-g/cm³ particles from *ts369*-infected cells were capsidlike structures. They failed to exclude uranyl

FIG. 5. Electrophoretic analysis of polypeptides present in particles isolated by centrifugation in a CsCl gradient. Particles within the indicated fractions from the gradients displayed in Fig. 4 were analyzed. IM, Particles of intermediate density (1.30 to 1.31 g/cm³); VIR, virion particles (1.34 g/cm³). An equal amount of radioactive protein from each sample was analyzed by electrophoresis. The molecular weights of radioactive marker proteins (left) and the identities of key virion polypeptides (right) are indicated.

acetate stain, consistent with their apparent lack of a normal complement of viral DNA (data not shown). At the permissive temperature, *ts369* accumulated mature virions, although, unlike results for wild-type virus, a band of 1.30- to 1.31-g/cm³ particles was also observed (data not shown).

The protein composition of 1.30- to 1.31-g/cm³ particles produced in *ts369*-infected cells was compared with that of *dl308/310* virions and empty capsids produced at 39.5°C. Fractions from the gradients shown in Fig. 4 were analyzed by electrophoresis (Fig. 5). Low-density intermediates isolated from *dl308/310*- or *ts369*-infected cells had very similar protein compositions. These intermediates consistently lacked proteins V and VII, the core proteins associated with virion DNA. This lack of core proteins and DNA is consistent with the low density of the intermediates.

The density of the particles that accumulated in *ts369*-infected cells at 39.5°C was higher than that of the empty capsids observed in *dl308/310* infections (compare Fig. 4A and B). Since no difference in protein components was observable between these two structures, we assayed for the presence of DNA in the *ts369* particles. Particles produced from 8.3×10^7 cells infected at a multiplicity of 100 PFU per cell with *ts369* at 39.5°C were pooled and purified by sequential banding on two CsCl gradients. The second gradient had a median density of 1.30 g/cm³ and separated the *ts369* intermediates into three distinct populations varying in density from 1.31 to 1.29 g/cm³. These populations were individually pooled, and DNA was isolated from the intermediates. Uncut or *Cla*I-*Kpn*I-cleaved DNA was subjected to DNA blot analysis, using the entire Ad5 genome as a probe DNA (Fig. 6). Lanes 1, 3, and 5 contain the digested DNAs from the high-, medium-, and low-density particles, respectively. The high-density population con-

tained the largest segment of viral DNA. The two fragments representing the leftmost regions of the adenovirus genome (arrows in Fig. 6) were found in the high-density particles, whereas only the very leftmost fragment was found in appreciable amounts in the medium-density particles. Very little DNA was recovered from the low-density particles. The lane containing RNA alone was included as a control to ensure that the carrier tRNA present in the DNA preparations did not contain material that could hybridize to the adenovirus DNA probe. From these results, we can conclude that the difference in density observed between the *ts369* particles and empty capsids resulted from small amounts of DNA found within the virion structures. The fact that only the leftmost portion of the viral genome was found within these capsids implies that the encapsidation of DNA observed is specific (adenovirus DNA is encapsidated in a polar fashion, beginning at the left end; 37) and not the result of nonspecific association of DNA with the virion structures.

Electron microscopic examination of *ts369*-infected cells confirmed the accumulation of virion intermediates. In an electron micrograph of a *dl308/310*-infected HeLa cell, the virions were visible as 70-nm hexagonal structures within the nucleus, most of which contained a densely stained core (Fig. 7A, inset). In contrast, the viral particles that had accumulated in the nucleus of *ts369*-infected cells appeared empty (Fig. 7B). The weakly stained *ts369* viral particles (best seen in the inset of Fig. 7B) were distinct from the irregular, heavily stained structures that resembled clumps of heterochromatin.

Particles that accumulate in *ts369*-infected cells at 39.5°C do not chase into virions at 32°C. A pulse-chase analysis of the capsids that accumulated in *ts369*-infected cells was performed to determine whether the virion structures were

FIG. 6. DNA blot analysis of Ad5-specific DNA present in incomplete particles isolated from *ts369*-infected 293 cells. Incomplete particles were purified through two sequential CsCl gradients. Three peaks of incomplete particles, ranging in density from 1.29 to 1.31 g/cm³, were obtained and designated low, medium, and high density. DNA was isolated from each population and subjected to DNA blot analysis, using the entire Ad5 genome as a ³²P-labeled probe DNA. Odd-numbered lanes received DNA that was cleaved with a mixture of *Cla*I and *Kpn*I; even-numbered lanes received uncut DNA. The two leftmost lanes contained *Cla*I-plus-*Kpn*I-cleaved viral DNA; the fragments that mapped to the two leftmost positions on the viral DNA are marked by arrows.

functional assembly intermediates. HeLa cells were infected at a multiplicity of 100 PFU per cell with *dl308/310* or *ts369* at 39.5°C. At 26 h postinfection, the cultures were labeled with a mixture of [³⁵S]methionine and [³⁵S]cysteine for 1 h at 39.5°C. After labeling, the cells were washed and placed in medium containing an excess of methionine for 3 h at 39.5°C, which allowed time for the labeled proteins to be incorporated into virion structures. After the 3-h chase, the cells were divided into three groups: one third of the cells was harvested immediately, one third was shifted to 32°C, and the final third was left at 39.5°C. After a further 12-h chase period, the remaining cells were harvested, disrupted, and loaded onto performed 1.2- to 1.4-g/cm³ cesium chloride gradients. A 12-h chase was chosen because it had been observed (Fig. 2) that after infection at 39.5°C, a 12-h recovery period at 32°C generated an approximately 10-fold increase in titer. This increase should be clearly observable

as a new mature virion peak on a CsCl gradient. Figure 8A shows the gradient profile from cells harvested immediately after the 3-h chase in a *dl308/310* infection. Few empty capsids were observed, and the majority of the labeled virions were mature. After 12-h chases at 39.5 and 32°C (Fig. 8B and 8C, respectively), there was little change in the gradient profile, although the titer of the peak virion fraction increased approximately twofold (data not shown). Figure 8D shows the gradient profile from a *ts369* infection harvested immediately after the initial 3-h chase. No mature virions were detected, and the peak of virion intermediates with a density of 1.30 g/cm³ was clearly present. After an additional 12-h chase at 39.5°C, the gradient profile for *ts369* infection (Fig. 8F) appeared essentially unchanged. After an additional 12-h chase at 32°C (Fig. 8F), a small peak that had the density of mature virions, 1.34 g/cm³, was observed. The virions formed at the permissive temperature led to an increase in titer of approximately 20-fold as compared with the titer of the virions isolated after the first harvest. The amount of radioactivity in the peak at 1.30 g/cm³ did not significantly decrease, however. Even after an 18-h chase at 32°C, this intermediate peak was not reduced, although the production of mature virions was clearly observable (data not shown). This finding implied that the mature virions produced during the chase were not formed by utilization of the empty particles produced at 39.5°C. If this had been the case, the intermediate peak would have been depleted. Rather, the new virions were probably formed by using labeled proteins from the protein pool within the infected cell. A chase period of 12 to 18 h should have been more than adequate to allow functional intermediates to proceed to mature virions. It takes about 1 h to observe assembly of newly synthesized proteins into virions (11, 20), and functional intermediates produced by an Ad5 fiber mutant have been quantitatively chased into virions within 4 h (3).

We conclude that the 1.30-g/cm³ particles produced in *ts369*-infected cells were not functional encapsidation intermediates. They could not be chased into mature virions.

Proteins that accumulate in *ts369*-infected cells at 39.5°C chase into virions at 32°C in the presence of cycloheximide. To determine whether the 52/55K proteins were reversibly inactivated by incubation at the nonpermissive temperature, a second pulse-chase experiment was performed in which a protein synthesis inhibitor was present after the temperature was shifted from 39.5 to 32°C. If the 52/55K proteins were inactivated at the nonpermissive temperature, virion production should not be observed during a chase at the permissive temperature in the presence of cycloheximide. Figure 9A displays the gradient profile of *ts369* particles obtained when, at 26 h after infection, cells were labeled for 1 h with [³²S]methionine and [³²S]cysteine and then chased for 3 h at 39.5°C. Figures 9B and C show the profiles obtained after 12-h chases at 32°C in the absence and presence of cycloheximide, respectively. In the presence of cycloheximide, total protein synthesis decreased to less than 5% of that observed in the absence of the drug (data not shown). Nevertheless, very similar amounts of radioactivity chased into particles with the density of virions in the absence or presence of drug (Fig. 9B and C). As observed in Fig. 8, the amount of radioactivity in the 1.30-g/cm³ particles did not decrease during chase periods at 32°C, again arguing that the 1.34-g/ml virion particles were generated from proteins that were not assembled into incomplete particles at the nonpermissive temperature.

Since assembly of virions occurred even though very little new 52/55K protein was synthesized during the 32°C chase

FIG. 7. Electron micrographs of HeLa cells infected with *ts369* or its wild-type parent. Spinner cultures were infected at a multiplicity of 20 PFU per cell with either *dl308/310* (A) or *ts369* (B) and harvested after 36 h at 39.5°C. Thin sections were prepared and stained with uranyl acetate and lead citrate. Bars in the main panels represent 1 μ m; those in the insets represent 0.25 μ m.

FIG. 8. Density gradient analysis of particles pulse-labeled in HeLa cells at 39.5°C and chased at either 39.5 or 32°C. Cells were infected at a multiplicity of 100 PFU per cell with either *dl308/310* (A to C) or *ts369* (D to F). Cultures were maintained at 39.5°C, labeled from 26 to 27 h after infection with a mixture of [³⁵S]methionine and [³⁵S]cysteine, and then harvested either after a chase period of 3 h at 39.5°C (A and D), after a chase period of 15 h at 39.5°C (B and E), or after a 3-h chase at 39.5°C plus a 12-h chase at 32°C (C and F). Particles were extracted and subjected to centrifugation on preformed 1.2- to 1.4-g/cm³ CsCl gradients. Gradients were fractionated, and radioactivity (○) was quantitated in a scintillation counter. The density of fractions (▲) was monitored by measuring the refractive index.

period in the presence of cycloheximide, it seems likely that the 52/55K proteins were reversibly inactivated at the non-permissive temperature.

DISCUSSION

This investigation was initiated to delineate the function(s) of the 52/55K proteins from the L1 region of adenovirus. We created a temperature-sensitive mutant (Table 1) that harbored a lesion within the open reading frame of these proteins (Fig. 1). This mutant was found to be blocked late in infection (Fig. 2) and accumulated a nonfunctional particle at the nonpermissive temperature (Fig. 4, 7, and 8). This particle displayed a density of 1.30 to 1.31 g/cm³ on a CsCl gradient (Fig. 4 and 8) and contained a small portion of the left end of the viral genome (Fig. 6). Presumably, these structures represent partially filled capsids that are stalled in the process of DNA encapsidation. The remainder of the viral DNA was likely sheared off during extraction and purification of particles by centrifugation (3).

Mutations within a variety of structural and nonstructural gene products are known to interfere with assembly of

virions. Mutations in structural polypeptides such as hexon, penton, or penton base can prevent accumulation of both mature virions and empty capsids (2, 9, 12, 33). Alterations in the IIIa or fiber structural polypeptides can prevent assembly of mature virions but allow accumulation of incomplete capsidlike particles (3, 8, 12, 23). Viruses with a defective L3-encoded protease gene accumulate DNA-containing virionlike particles that have a series of unprocessed polypeptides (38). Since normal proteolytic cleavage events are not carried out, the particles are noninfectious. Mutants with lesions in the adenovirus DNA polymerase have been reported to accumulate incomplete capsidlike particles when shifted to the nonpermissive temperature (9). Finally, we have recently observed that *sub100*, an Ad5 variant with a temperature-sensitive mutation within the terminal protein gene (14) whose gene product functions to prime viral DNA replication, accumulates incomplete capsidlike particles when propagated at the permissive temperature (T. Hasson, J. Schaack, and T. Shenk, unpublished observation). Thus, mutations within major structural proteins, the viral protease, or gene products that function during initiation of DNA

FIG. 9. Density gradient analysis of particles pulse-labeled in HeLa cells at 39.5°C and chased at 32°C in the presence or absence of cycloheximide. Methods were as described in the legend to Fig. 8. (A) Harvested after a 3-h chase at 39.5°C; (B) harvested after a 3-h chase at 39.5°C plus a 12-h chase at 32°C; (C) as in panel B, with cycloheximide (20 µg/ml) present in the chase medium during the 32°C incubation. Symbols: ○, radioactivity; ▲, density.

replication can interfere with assembly of mature virus particles.

ts369 appears to represent a unique class of assembly mutant. It differs from protease mutants in that a different type of particle accumulates within *ts369*-infected cells under nonpermissive conditions. Unlike DNA polymerase and terminal protein mutants, viral DNA replication is normal within *ts369*-infected cells at the nonpermissive temperature. In contrast to mutants carrying lesions in major structural polypeptides, the 52/55K proteins are not major constituents of virions. In fact, these proteins are not known to be associated with the virion. Lewis et al. (25) and Lucher et al. (26) were unable to detect the 52/55K proteins in virion preparations by immunoprecipitation. We have also attempted to demonstrate the presence of these proteins in purified virions by immunoprecipitation using a monospecific polyclonal antibody (26), but without success (data not shown). These results do not rule out the presence of the 52/55K proteins in virions; if they were present, however, each virion contained very few molecules.

Studies of mutant viruses and analysis of the kinetics with which polypeptides enter incomplete capsids and mature virions have provided a rough outline of the viral assembly process. Assembly appears to proceed by the initial formation of an empty capsid, followed by insertion of DNA (32, 35; reviewed in reference 30). Capsid formation might occur spontaneously in the absence of viral DNA, and a subsequent recognition between capsid and DNA might initiate insertion. Alternatively, capsid formation could be initiated in association with a viral DNA molecule. Either mechanism requires a recognition signal on the viral DNA. This signal probably involves proteins assembled at a *cis*-acting packaging sequence centered at about 260 base pairs from the left end of the viral chromosome (17–19, 37) and at the origin of DNA replication, explaining the block to assembly in DNA polymerase and terminal protein mutants discussed above. The *cis*-acting packaging sequence functions only near an end of the viral chromosome (18), raising the possibility that an interaction occurs between factors at that site and the origin of DNA replication. The mechanism whereby the viral DNA enters the capsid is not known; a requirement for ongoing DNA replication is controversial (7, 40). The L3-encoded protease functions late in the process; the proteolytic cleavages presumably stabilize the structure and render the particle infectious.

What is the role of the 52/55K proteins in encapsidation? Intermediates can be isolated from *ts369*-infected cells that appear to represent capsids associated with the left end of the viral chromosome. It is tempting to speculate that the 52/55K proteins function fairly late in the assembly process, after the capsid-DNA recognition event and before completion of insertion and compaction of the DNA within the capsid. The 52/55K proteins might be involved in the insertion-compaction phase of encapsidation. However, they might function earlier in the assembly process, and an earlier failure, e.g., formation of a defective capsid, would lead to a block during the insertion-compaction step. The fact that the *ts369* mutation is recessive (data not shown) militates against but does not rule out this possibility. Thus, although it is clear that proper function of the L1 52/55K proteins is essential for successful encapsidation, the precise role they play in the process remains uncertain.

Finally, it is worth mentioning that the 52/55K proteins might carry out functions in addition to the packaging role we have delineated through analysis of *ts369*. These proteins are synthesized relatively early in comparison with other gene products encoded by the major late transcription unit (26). Perhaps they mediate additional functions earlier in the adenovirus replication cycle that are not disrupted by the mutation in *ts369*.

ACKNOWLEDGMENTS

We thank Elaine Lenk for assistance in electron microscopy and Maurice Green for a gift of antibody to the 52/55K proteins.

This work was supported in part by grant MV-45 from the American Cancer Society. T.B.H. is and P.D.S. was a predoctoral trainee of the National Cancer Institute (Public Health Service grant CA 09528), and D.A.O. is an American Cancer Society research fellow. The sabbatical stay of W.D. at Princeton University was supported by the Deutscher Akademischer Austauschdienst, Bonn-Bad Godesberg, Federal Republic of Germany. T.S. was an American Cancer Society research professor.

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