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Morphogenesis of Human Adenovirus Type 2 Studied with Fiber- and Fiber and Penton Base-Defective Temperature-Sensitive Mutants

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The nature, polypeptide composition, and antigenic composition of the particles formed by six human adenovirus type 2 temperature-sensitive (*ts*) mutants were studied. *ts*115, *ts*116, and *ts*125 were phenotypically fiber-defective mutants, and *ts*103, *ts*104, and *ts*136 failed to synthesize detectable amounts of fiber plus penton base at 39.5°C. The mutants belonged to five complementation groups, one group including *ts*116 and *ts*125. Except for *ts*103 and *ts*136, the other mutants were capable of producing particles at 39.5°C. *ts*116 and *ts*125 accumulated light assembly intermediate particles (or top components) at nonpermissive temperatures, with few virus particles. The sodium dodecyl sulfate polypeptide pattern of *ts*116- or *ts*125-infected cells, intermediate particles, and virus particles showed that polypeptide IV (fiber) was smaller by a molecular weight of 2,000 than that in the wild-type virion and was glycosylated. In fiber plus penton base-defective *ts*104-infected cells, equivalent quantities of top components and viruses with a buoyant density (ρ) of 1.345 g/ml (ρ = 1.345 particles) were produced at 39.5°C. These ρ = 1.345 particles corresponded to young virions, as evidenced by the presence of uncleaved precursors to proteins VI, VIII, and VII. These young virions matured upon a shift down. Virus capsid vertex antigenic components underwent a phase of eclipse during their incorporation into mature virus particles. No antigenic penton base or IIIa was detected in intermediate particles of all the *ts* mutants tested. Only hexon and traces of fiber antigens were found in *ts*104 young virions. Penton base and IIIa appeared as fully antigenically expressed capsid subunits in mature wild-type virions or *ts*104 virions after a shift down. The *ts*104 lesion is postulated to affect a regulatory function related in some way to penton base and fiber overproduction and the maturation processing of precursors PVI, PVIII, and PVII.

The adenovirus assembly pathway proceeds from soluble capsid components via multiple steps involving assembly intermediate (IM) particles separable from mature virions in CsCl gradients and identifiable by sodium dodecyl sulfate (SDS)-polyacrylamide gel patterns (5, 12). Among these IM particles, there are those devoid of 31S viral DNA, the so-called top components, banding at a buoyant density (ρ) of 1.30 to 1.31 g/ml in CsCl (4, 5, 12), heavy IM particles of ρ = 1.37 g/ml (3), and immature, or "young virions" (YV [5, 12]).

Temperature-sensitive (*ts*) mutants are invaluable tools for determining the function of human adenovirus (HAd) proteins in virion morphogenesis (11). Sets of HAd *ts* mutants have been selected and characterized in several laboratories and include HAd2 (1, 14, 21), HAd5 (7, 35), HAd7 (8), HAd12 (18, 27), and HAd31 (32).

The HAd2 penton base and fiber projection is known to be a weak point in the virus icosahedron (17). In the present study, the role of the vertex subunits in HAd2 capsid formation was studied with the aid of six *ts* mutants, three altered in a single component (fiber) and three altered in a pair of two capsid components (fiber and penton base).

MATERIALS AND METHODS

Cells and viruses. Wild-type (WT) HAd2, originally obtained from J. F. Williams (Carnegie-Mellon Institute, Pittsburg, Pa.), was grown on KB cells cultured in suspension (2.0×10^5 to 5.0×10^5 cells per ml) in Eagle Spinner medium supplemented with 5% horse serum. HeLa cells were grown in monolayers in Eagle minimal essential medium supplemented with 10% calf serum. Virus was titrated by the plaque assay or by the fluorescent focus unit (FFU) assay (25) on a HeLa cell monolayer at 37°C.

ts mutants of HAd2 (block no. 101 to 200 [21]) were isolated after nitrous acid treatment of a WT stock,

which underwent two cycles of plaque purification at 37°C. Thirty-nine *ts* mutants were thus obtained from 400 clones isolated at random (21).

Antisera. Polyspecific rabbit antiserum against HAd2 virion proteins was obtained by multiple subcutaneous injections of HAd2 particles purified by two cycles of equilibrium centrifugation in CsCl (20, 21). Monospecific antisera against hexon, penton base, fiber, or IIIa were obtained by injection of corresponding antigen-antibody precipitates freshly formed in agarose gel after two-dimensional immunoelectrophoresis (2; P. Lemay, M. L. Boudin, M. Milleville, and P. Boulanger, Virology, in press).

Complementation tests. HeLa cell monolayers were doubly infected at an input multiplicity of 5 FFU of the two *ts* mutants per cell. After 2 h of adsorption at 33°C, unadsorbed virus was rinsed off, and the cells were treated for 30 min with an anti-HAd2 virion serum at a dilution of 1:100. The cells were rinsed again and incubated at 39.5°C for an additional 40 h. Control cultures were singly infected in parallel with 10 FFU of each mutant per cell. At the end of the incubation period, the cells were scraped into the culture medium and disrupted by three cycles of freezing and thawing, and virus was titrated with the FFU assay at 33°C. The complementation index was given as the ratio of yield of the double infection to that of the higher of the two single infections at 39.5°C, expressed as FFU per milliliter. Complementation is usually considered as positive when the complementation index is at least 10 (35).

Immunological characterization of *ts* mutants. HAd2 *ts* mutants were serologically characterized by quantitation in crossed immunoelectrophoresis of the soluble antigens produced by the infected cells at 39.5°C compared with 33°C and with the WT at both temperatures (20, 21).

Quantitative and qualitative analyses of hexon, fiber, penton base, and IIIa antigens were performed in crossed immunoelectrophoresis of deoxycholate (DOC)-disrupted virions (2, 2a, 24; Lemay et al., Virology, in press). Virus particles were suspended in 0.005 M Tris-hydrochloride buffer, pH 7.8, and heated with 0.5% sodium DOC at 56°C until disappearance of the opalescence (usually 60 to 90 s). This treatment did not damage the hexon, fiber, and IIIa, but penton base was separated from the fiber projection (2; Lemay et al., Virology, in press).

Pulse-chase of infected cells. Pulse-chase labeling experiments were performed in KB cells (6×10^6 cells per ml) in methionine-deprived medium. L-[^{35}S]methionine (50 μCi/ml) was added for 20 min at different times postinfection at 39.5°C (nonpermissive temperature) or at 33°C (permissive temperature). The cells were harvested just after the pulse or chased by dilution to 3×10^5 cells per ml in medium prewarmed to the required temperature and containing 10 times the normal concentration of cold methionine. L-[^{35}S]methionine-labeled virus-induced and virion proteins were analyzed in SDS-containing polyacrylamide gels.

Analytical SDS-polyacrylamide gel electrophoresis. Samples were dissolved in an equal volume of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8, containing 6 M urea, 4% SDS, and 10% 2-mercaptoethanol) and heated for 2 min at 100°C. Polypeptides were analyzed in an SDS-containing 17.5% polyacrylamide gel (acrylamide/bisacrylamide ratio of 50:0.235) overlaid by a 5% spacer gel (acrylamide/bisacrylamide ratio of 50:1.33) in the discontinuous buffer system of Laemmli (16). The gels were stained with Coomassie brilliant blue R-250, dried under vacuum, and exposed to Kodak Kodirex film.

Extraction and purification of virus particles and top components. Infected cells, harvested late in the infectious cycle (20 to 30 h after infection), were extracted with Freon 113 as previously described (3), and mature or YV (ρ = 1.345 g/ml) and light IM particles or top components (ρ = 1.30 to 1.31 g/ml) were separated in self-generating CsCl gradients (3).

Cell fractionation and extraction of assembly IM and virus particles. The method of separation of cytoplasmic and nuclear fractions and the extraction of particles from nuclei have been described in detail elsewhere (3, 4). Assembly IM and virus particles were isolated on a linear (25 to 40%) sucrose gradient: virions sediment at 750S, and assembly IM particles sediment at about 600S (3, 4).

The different classes of particles were purified further in self-generating CsCl gradients after fixation with a cleavable diimido ester (dimethyl-4,4'-dithiobisbutyrimidate [3, 4]).

Extraction and isolation of adenovirus core. Virions or IM particles were disrupted with 0.5% DOC as described above. The different subviral entities were separated in a discontinuous sucrose gradient (40 to 70%, 3-ml total volume; overlaid by a 5 to 20% gradient, 8-ml total volume; made in 0.02 M sodium borate buffer, pH 8.0, containing 1 M NaCl and 0.001 M EDTA). The gradients were centrifuged at 35,000 rpm for 2 h at 4°C in a Beckman SW41 rotor. Virus cores sediment at 180 to 200S, groups of nine hexons sediment at 50 to 60S, and isolated capsomers sediment at between 6 and 12S (2a).

Chemicals and radioisotopes. Sodium DOC was purchased from BDH, Poole, United Kingdom. A 10% solution (wt/vol) was made in water and dialyzed in the cold against distilled water. The dialyzable fraction obtained was lyophilized and used for virus disruption.

Cycloheximide (CH) was purchased from Boehringer, Mannheim, Federal Republic of Germany, and used at a concentration of 20 μg/ml of cell culture. Puromycin (Sigma Chemical Co., St. Louis, Mo.) was used at 10 μg/ml.

Methyl-4-mercaptobutyrimidate hydrochloride (Pierce Chemical Co., Rockford, Ill.) was used at a concentration of 3 mg/ml in medium devoid of primary amine. The reaction of amidination of proteins was allowed to proceed for 30 min at 4°C. Disulfide bridges were induced by dialysis against phosphate-buffered saline containing 0.1 M H_2O_2 (3). The induced disulfide cross bridges could be cleaved by an excess of reducing agent (e.g., 2-mercaptoethanol or dithiothreitol).

Bovine serum albumin (2× crystallized; Sigma Chemical Co.) was used as a standard for the protein assay (19).

L-[^{35}S]methionine (700 to 800 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom, and [^{3}H]thymidine (25 Ci/mmol) was from the Commissariat à l'Energie Atomique,

Saclay, France. Pulse-labeling of viral proteins and DNA was performed at 50 μCi of radioisotopes per ml for 20 min at a late time after infection (16 h postinfection). [^{3}H]glucosamine (Commissariat à l'Energie Atomique) with a specific activity of 12 Ci/mmol was used at 50 μCi/ml in prolonged labeling (16 to 24 h after infection).

RESULTS

Complementation. As shown in Table 1, *ts*103 strongly complemented *ts*104, *ts*115, *ts*116, *ts*125, and *ts*136. *ts*104 also complemented *ts*125 and *ts*136, and *ts*115 complemented *ts*116, *ts*125, and *ts*136. There was no ambiguity either for *ts*116 and *ts*136 or for *ts*125 and *ts*136, if a limit of 10 was chosen as a positive complementation index. A complementation index of 3.6 was found between *ts*104 and *ts*115, a complementation index of 6.7 was found between *ts*104 and *ts*116, and a complementation index of 7.2 was found between *ts*116 and *ts*125. This suggested that there were three complementation groups, one group including *ts*103, the second group including *ts*136, and the third group including *ts*104, *ts*115, *ts*116, and *ts*125.

However, *ts*115 and *ts*116 complemented each other, as did *ts*115 and *ts*125. In addition, the biological properties of *ts*104, *ts*115, and *ts*116 were different as shown below. In contrast, *ts*116 and *ts*125, which complemented each other poorly, could not be distinguished serologically or biochemically. Therefore, five complementation groups were suggested, two groups including the three fiber-defective mutants, *ts*115 in one group and both *ts*116 and *ts*125 in the other, and three groups corresponding to each of the three penton base and fiber-defective mutants *ts*103, *ts*104, and *ts*136.

All of the mutants, except *ts*115, were strongly complemented by HAd2 *ts*112, a mutant defective in late functions essential for a normal virus maturation (4). *ts*112 showed a serological pattern at 39.5°C similar to the WT pattern, with respect to the production of hexon, penton base, penton, and fiber (21). This result suggests that *ts*115 is a double mutant.

Serological characterization. Five of the six mutants studied have previously been characterized serologically by the two-dimensional immunoelectrophoresis pattern of cells infected with the *ts* mutants at 39.5°C (21). However, careful reexamination of the two-dimensional patterns of *ts*104 and *ts*116, previously catalogued in the same complementation group, C, indicated that the two mutants should be classified in two separate groups, C and L: *ts*116 appeared fiber defective, as did *ts*115 (group E), and presented the same polypeptide pattern as did *ts*125 (group L). *ts*104 was phenotypically penton base and fiber defective (21).

The new mutant *ts*136 failed to synthesize an excess of penton base and fiber in the infected cell and resembled the *ts*103 and *ts*104 phenotype. The biological properties of these mutants are summarized in Table 2.

***ts* mutant polypeptides in infected cells.** KB cells were infected with the WT or a *ts* mutant at 10 FFU/cell at 39.5°C and pulse-labeled for 1 h at 16 h after infection. The cell culture was divided into three portions, one being arrested just after the pulse, one being chased for 8 h at 39.5°C, and the third one being chased for 24 h at 33°C. Virus-coded polypeptides were analyzed in SDS-polyacrylamide gels.

Figure 1 shows that, except for *ts*116 and *ts*125, there was no visible cleavage of the precursors to virion proteins VI, VIII, and VII during the chase at 39.5°C. The precursor PVI of *ts*115 migrated nearer PVIII than in the WT or in the other *ts* mutants, with an apparent molecular weight of 26,500 instead of 27,000. The same difference in molecular weight was observed between polypeptide VI of *ts*115 virions produced at 33°C and polypeptide VI of the WT virus particles (see Fig. 9).

In *ts*116- and *ts*125-infected cells, the apparent

TABLE 1. *Complementation indices between fiber-defective and fiber and penton base-defective ts mutants of HAd2*

Mutant	Complementation index						
	*ts*103	*ts*104	*ts*115	*ts*116	*ts*125	*ts*136	*ts*112[a]
*ts*103 (F)	—[b]	125	67	322	360	75	75
*ts*104 (C)		—	3.6	6.7	37	56	50
*ts*115 (E)			—	20	18	26	3.9
*ts*116 (L)				—	7.2	20	52
*ts*125 (L)					—	10	135
*ts*136 (M)						—	50
*ts*112[b] (I)							—

[a] HAd2 *ts*112 is blocked in late functions essential for a normal virion maturation (4). Its serological pattern at 39.5°C resembles that of the WT (21). Letters within parentheses are complementation groups.

[b] —, Dash indicates a complementation index of 1.

TABLE 2. *Soluble capsid antigens and particles produced by fiber-defective and fiber and penton base-defective ts mutants of HAd2 at a late stage after infection at 39.5°C*[a]

Mutant	Complementation group	Soluble antigens				Particles	
		Hexon	Penton base	Fiber	IIIa	Top components	Virions
*ts*103	F	+	±	±	±	0	0
*ts*104	C	+	±	±	±	+	+ (YV)
*ts*115	E	+	+	0	±	++	± (mature)
*ts*116	L	+	+	±	±	++	± (mature)
*ts*125	L	+	+	±	±	++	± (mature)
*ts*136	M	+	0	0	0	0	0

[a] ++, Increased amount, compared with the WT; +, same amount as in the WT; ±, trace amount; 0, not detectable. Top components: light particles banding at 1.30 to 1.31 g/ml in CsCl.

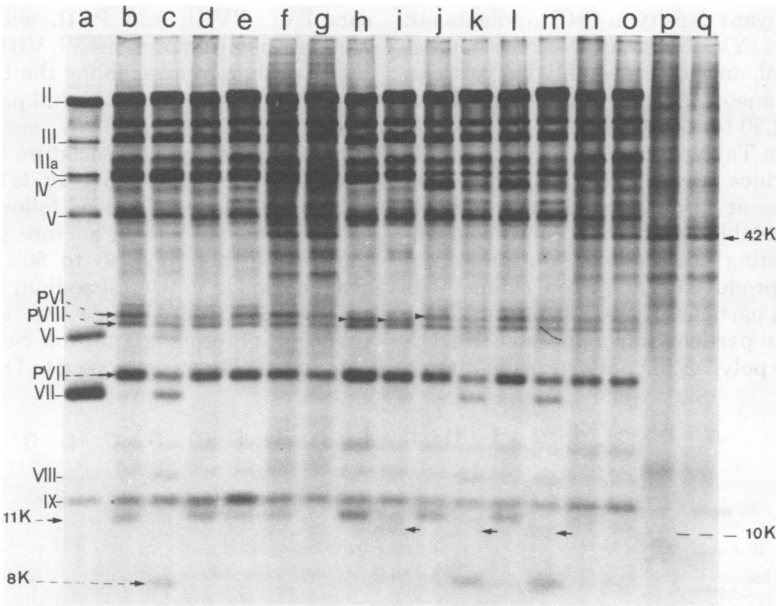

FIG. 1. *SDS-polyacrylamide gel autoradiogram of polypeptides of WT-, ts mutant-, and mock-infected KB cells at 39.5°C. The cells were pulse-labeled with [^{35}S]methionine for 1 h at 16 h after infection (b, d, f, h, j, l, n, and p) and chased for 8 h at 39.5°C (c, e, g, i, k, m, o, and q). (a) WT HAd2 virion; (b and c) WT-infected cells; (d and e) ts103; (f and g) ts104; (h and i) ts115; (j and k) ts116; (l and m) ts125; (n and o) ts136; (p and q) mock-infected cells. A 60,000-molecular-weight (60K) polypeptide is visible in place of polypeptide IV (62K) in ts116 (j)- and ts125 (l)-infected cells. Cleavage of PVII into VII occurs during the chase in WT-, ts116-, and ts125-infected cells, whereas an 11K polypeptide disappears and an 8K polypeptide appears. The label decreases in the 60 to 62K polypeptides during the chase at 39.5°C in ts115, ts116, and ts136, whereas a 10K polypeptide becomes visible. Note that actin polypeptide synthesis (42K) is not completely inhibited in ts104 and ts136 infection.*

molecular weight of polypeptide IV was 2,000 lower than that in the WT or in other *ts* mutants. In these two mutants, some cleavage of PVII to VII occurred during the chase of 39.5°C, although at a slower rate than that in the WT, a polypeptide with a molecular weight of 11,000 disappeared, and a polypeptide band with a molecular weight of 8,000 appeared. In *ts*115, *ts*116, *ts*125, and *ts*136, the fiber polypeptide (IV) decreased during the chase at 39.5°C, and an extra polypeptide band with a molecular weight of 10,000 appeared.

During a 24-h chase period at 33°C, the polypeptide patterns of the *ts* mutants were similar to that of the WT, with cleavage of the precursors PVI, PVIII, and PVII. However, the lesion of *ts*116 and *ts*125 was still apparent at 33°C, with a polypeptide with a molecular weight of 60,000 in place of the fiber polypeptide with a molecular weight of 62,000 (Fig. 2).

The possibility of a defect in the glycosylation process of the fiber protein at 39.5°C was examined by labeling infected cells with [^{3}H]glucosamine for 8 h at 16 h after infection. SDS-polyacrylamide gel analysis revealed that the *ts* mutants studied possessed a glycosylated fiber polypeptide, the same as that of the WT (13). In *ts*116 and *ts*125, the 60,000-molecular-weight polypeptide replacing polypeptide IV fiber was also glycosylated (Fig. 3).

Particles produced at the end of the infectious cycle at 39.5°C. Cells infected at 10 FFU/cell at 39.5°C were harvested 30 h after infection, and virus particles or IM particles or both were extracted with fluorocarbon and separated by buoyant density in CsCl gradients (4): virus particles (YV and mature virions) band at ρ = 1.345 g/ml, and light assembly IM particles or top components devoid of 31S viral DNA band at ρ = 1.30 to 1.31 g/ml (12).

As shown in Table 2, mutants *ts*103 and *ts*136 did not produce any detectable particles at 39.5°C, neither at ρ = 1.345 g/ml nor at ρ = 1.30 g/ml. Only soluble components were overproduced, consisting essentially of hexon capsomers. *ts*115 produced mainly top components and few virus particles of ρ = 1.345 g/ml. These ρ = 1.345 g/ml particles had a normal 31S DNA and the same polypeptide pattern as did mature WT virions (data not shown). These particles might result from a certain degree of leakiness of this mutant (21).

*ts*116 and *ts*125 also produced mainly top components at 39.5°C and a minor population of virions of ρ = 1.345 g/ml. These virions possessed a 60,000-molecular-weight polypeptide in place of the 62,000-molecular-weight (IV) polypeptide normally present in the WT (see Fig. 8f).

In contrast to the other mutants, *ts*104 produced equivalent quantities of top components and ρ = 1.345 g/ml virus particles. When analyzed in SDS-polyacrylamide gels, these ρ = 1.345 g/ml particles showed uncleaved precursors PVI, PVIII, and PVII, with only trace amounts of virion proteins VI, VIII, and VII (see Fig. 7b), thereby resembling the HAd2 *ts*1 mutant (33). These ρ = 1.345 g/ml particles might, therefore, be considered YV, according to the generally accepted nomenclature (5, 12).

Absence of DOC core in *ts*104 YV. The *ts*104 YV were purified as follows. The peak sedimenting at 750S in sucrose gradients was purified further in a 40 to 50% metrizamide gradient made in 0.02 M sodium borate buffer, pH 8.0 (10). Metrizamide was eliminated by exclusion chromatography on Sephadex G-50. The *ts*104 YV were subjected to DOC treatment

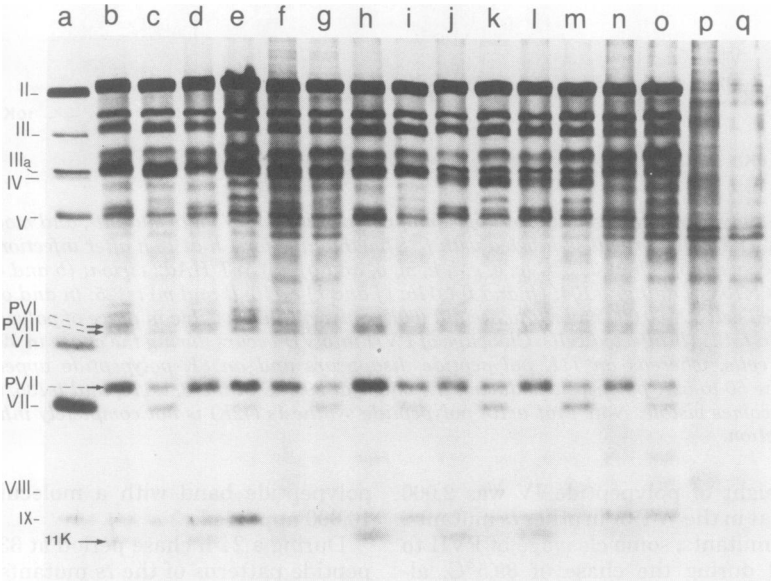

FIG. 2. *SDS-polyacrylamide gel autoradiogram of temperature-shift experiments. WT-, ts mutant-, or mock-infected KB cells were pulse-labeled for 1 h at 39.5°C at 16 h after infection (b, d, f, h, j, l, n, and p) and chased for 24 h at 33°C (c, e, g, i, k, m, o, and q). (a) WT HAd2 virion; (b and c) WT-infected cells; (d and e) ts103; (f and g) ts104; (h and i) ts115; (j and k) ts116; (l and m) ts125; (n and o) ts136; (p and q) mock-infected cells. Processing of PVII into VII and disappearance of an 11,000-molecular weight (11K) polypeptide occur upon a shift down in every infected-cell sample.*

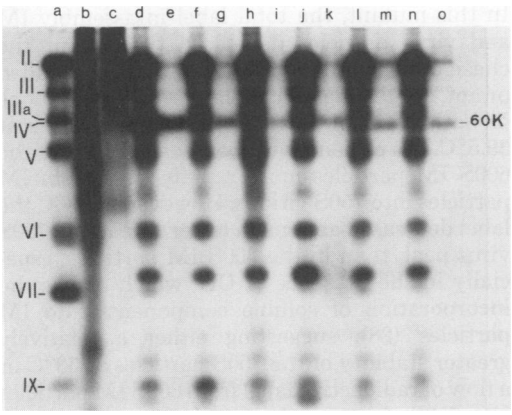

FIG. 3. *Glycosylation pattern of adenovirus-infected cell polypeptides. KB cells were mock infected (b and c) or infected with the WT (d and e), ts103 (f and g), ts104 (h and i), ts115 (j and k), ts116 (l and m), and ts125 (n and o). Labeling was performed with [^{35}S]methionine (b, d, f, h, j, l, and n) or [^{3}H]glucosamine (c, e, g, i, k, m, and o) for 8 h at 16 h after infection. (a) Control HAd2 virion. Polypeptide IV (fiber) has been found to be labeled with [^{3}H]glucosamine (13). Note the lower molecular weight of ts116 and ts125 late major glycopolypeptide: 60,000 (60K) instead of 62K. The ^{3}H label was revealed by fluorography in PPO (2,5-diphenyloxazole)-impregnated gels.*

at 56°C, and the resulting virus lysate was analyzed on a sucrose gradient as described above. Groups of nine hexons were visible at 50 to 60S, and isolated capsomers were visible at between 6 and 12S, but no viral cores were obtained from these particles. A peak of [^{3}H]thymidine-labeled viral DNA was found at 31S in the gradient (Fig. 4a). After a shift down to 33°C, the *ts*104 YV matured, as evidenced by the cleavages of PVII into VII and of PVI and PVIII into VI and VIII, respectively. DOC treatment of the temperature-shifted *ts*104 YV revealed the presence of a DNA-containing DOC core, with an apparent sedimentation coefficient of 180 to 200S (Fig. 4b).

Antigenicity of capsid proteins within the particles produced by *ts* mutants. The different classes of particles produced by mutants *ts*104, *ts*115, *ts*116, and *ts*125 were disrupted with DOC, analyzed in two-dimensional immunoelectrophoresis against antivirion serum, and compared with the DOC-disrupted WT pattern. The results are summarized in Table 3 and illustrated in Fig. 5. The top components, or light IM particles, of the mutants studied and of the WT exhibited no antigenically active penton base and IIIa: hexon was the only major detectable capsid antigen. Fiber antigen was present in a normal amount in WT top components, but was found only in trace amounts in top components of *ts*104. *ts*104 YV showed no penton base but traces of fiber antigen. However, all particles possessed the corresponding polypeptides visible in SDS-polyacrylamide gels, viz., III, IIIa, and IV (see Fig. 7 and 8).

In *ts*116- and *ts*125 top components and mature virions, no antigenic fiber was detectable, and the SDS polypeptide pattern showed the absence of the fiber polypeptide unit with a molecular weight of 62,000 and replacement by a 60,000-molecular-weight polypeptide (see Fig. 8f). As in WT top components, light particles of

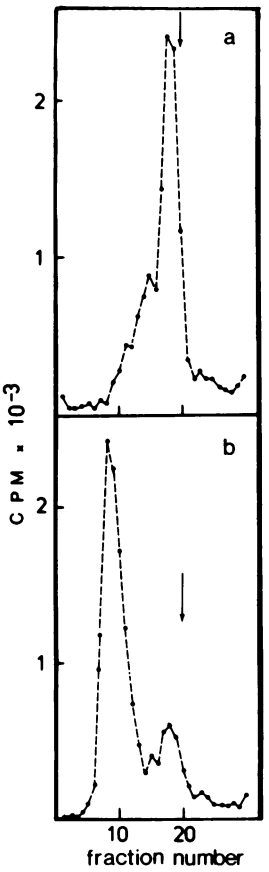

FIG. 4. *Core analysis of ts104 particles after a shift down. KB cells were infected with ts104 at 39.5°C, pulse-labeled for 20 min at 16 h postinfection with [^{3}H]thymidine (50 μCi/ml), and chased at 39.5°C for 3 h. One sample was withdrawn after the chase; virus particles were extracted, disrupted with DOC, and analyzed in a discontinuous sucrose gradient (a). The other sample (b) was chased for 16 h at 33°C in the presence of CH (20 μg/ml), and particles were treated as in (a). The arrow indicates the position of the 31S virus DNA marker.*

ρ = 1.315 g/ml accumulated by *ts*112 at restrictive temperatures (4) contained only hexon and fiber antigens. Antigenic penton base appeared only in mature virions produced normally by the WT or in minor quantities by *ts*125 at 39.5°C (Fig. 5).

Pulse-chase and temperature-shift experiments. An inhibition of protein synthesis in infected cells has been shown to block the formation of IM particles from soluble virus components (28), but does not prevent the maturation of IM particles and YV into mature virions (4). A shift down to 33°C in the presence of CH was therefore used to determine whether the IM particles or YV assembled at 39.5°C were capable of evolving into normal mature virions. Infected cells were pulse-labeled for 20 min at 39.5°C at 16 h after infection and chased with cold methionine for 2 h at 39.5°C. The cell culture was then divided into six portions. One was immediately processed for virus particle extraction (sample 1). One was further chased for 5 h at 39.5°C (sample 2). One was chased at 39.5°C for 1 h in the presence of CH (sample 3). A fourth one was chased for 1 h at 39.5°C with CH, then shifted down to 33°C, and incubated at this temperature for an additional period of 21 h with CH (sample 4). Sample 5 was chased at 33°C for 22 h in the presence of CH, and the last sample was chased at 33°C for 22 h without CH (sample 6). A scheme of this experiment is shown in Fig. 6.

The IM and virus particles were extracted from each cell sample and analyzed on sucrose density gradients. IM particles sediment as a peak of 600S, whereas virions sediment at 750S (3, 5).

Table 4 shows the results obtained with *ts*104.

TABLE 3. *Antigenicity of the major capsid proteins of DOC-treated virus particles and assembly intermediates of HAd2 ts mutants and the WT*

Virus	Type of particles analyzed	Capsid proteins[a]			
		Hexon	Penton base	Fiber	IIIa
*ts*104	Top components	+	0	±	0
*ts*104	YV	+	0	±	0
*ts*115	Top components	+	0	0	0
*ts*116	Top components	+	0	0[b]	0
*ts*125	Top components	+	0	0[b]	0
*ts*125	Mature virions	+	+	0[b]	±
*ts*112[c]	Top components	+	0	+	0
WT	Top components	+	0	+	0
WT	Mature virions	+	+	+	+

[a] +, Same amount of antigen as in the DOC-treated WT virion; ±, trace amount; 0, no detectable antigen.

[b] Presence of a 60,000-molecular-weight polypeptide in place of the fiber polypeptide of 62,000 molecular weight.

[c] HAd2 *ts* mutant defective in virion morphogenesis and accumulating light assembly IM particles (4).

In this mutant, the total label in assembly IM and virus particles decreased throughout the chase at 39.5 and 33°C, whether CH was added or not, suggesting a significant breakdown of one or both types of particles. During the chase at 39.5°C, the radioactive label seemed to enter the 600S IM particles more rapidly than from IM particles into 750S virions. However, at 33°C the label decreased at a much lesser rate in the 750S virus peak than in the 600S IM particles, especially in the presence of CH, which blocks the incorporation of soluble components into IM particles (28), suggesting either a relatively greater stability of the 750S particles at 33°C or a flow of radioactive label from 600S IM particles into 750S virions, or both.

*ts*103 and *ts*136 showed no detectable peak of labeled IM and virus particles at 39.5°C. *ts*115 showed only a peak of IM particles, with no occurrence of 750S virions, even after a shift down (data not shown). Both *ts*116 and *ts*125 assembled few 750S virions at 39.5°C, with an apparently normal maturation at either 39.5 or 33°C, as shown by the polypeptide pattern (see Fig. 8). At 33°C, the peak of 750S particles increased. The maturation process occurred also in the presence of CH at 39.5 and 33°C (data not shown).

Polypeptide pattern of particles obtained in pulse-chase and temperature-shift experiments. The 750S and 600S peaks of each of the six gradients shown in Fig. 6 were analyzed in SDS-polyacrylamide gels. The polypeptide compositions of *ts*104 and *ts*125 IM and virus particles are shown in Fig. 7 and 8. *ts*104 YV accumulated at 39.5°C were capable of maturation upon a shift down, as evidenced by the processing of precursors PVI, PVIII, and PVII and the appearance of polypeptide XI. This maturation processing also occurred when infected cells were shifted down to 33°C in the presence of CH or puromycin, suggesting that the putative virus-coded endopeptidase (33) was already synthesized but inactivated at 39.5°C (Fig. 7).

*ts*125, as well as *ts*116, showed an abnormal polypeptide pattern of assembly IM and virus particles, with a 60,000-molecular-weight polypeptide in place of the 62,000-molecular-weight fiber polypeptide. The *ts*116 and *ts*125 virions which matured at 33°C in the presence or absence of CH also contained the 60,000-molecular-weight polypeptide in place of polypeptide IV (Fig. 8 and 9).

DISCUSSION

The present study confirms previous reports on adenovirus (6, 26, 36) or bacteriophage systems (22), showing that fiber-defective mutants can assemble particles under restrictive condi-

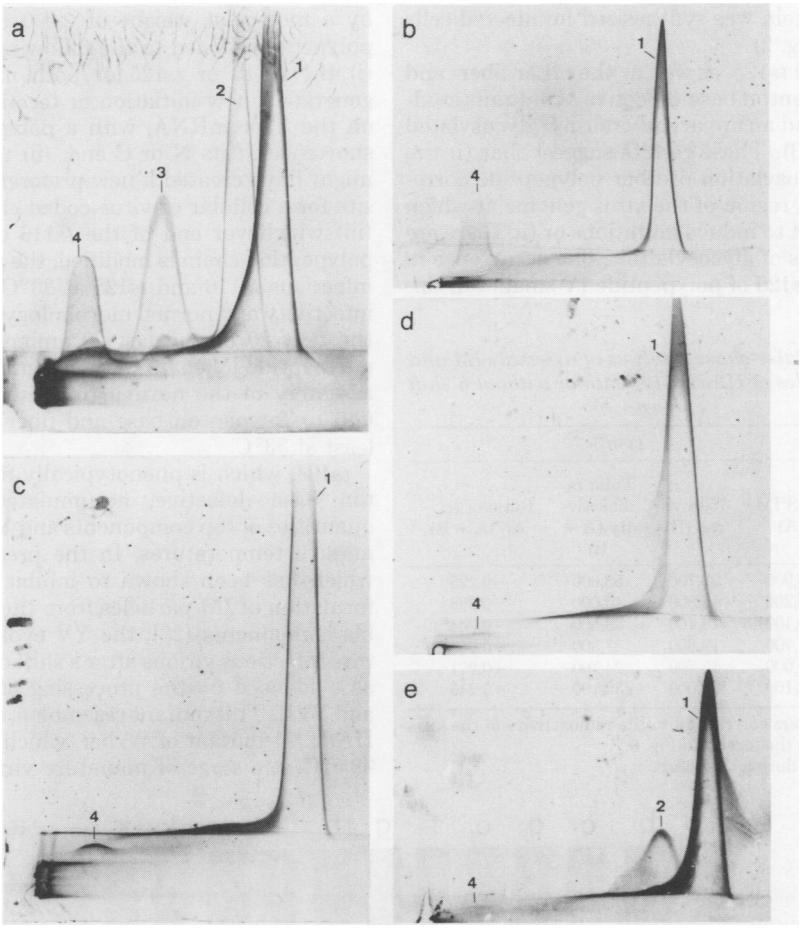

FIG. 5. *Two-dimensional immunoelectrophoretic analysis of antigenic components of WT and ts mutant particles. The particles were disrupted with 0.5% DOC before two-dimensional analysis. (a) WT mature virions; (b) WT top components (or assembly IM particles of ρ = 1.315 g/ml); (c) ts104 YV of ρ = 1.345 g/ml; (d) ts104 top components; (e) ts125 virions of ρ = 1.345 g/ml. The peaks of immune precipitates have been identified previously as follows: (1) hexon; (2) penton base; (3) protein IIIa; (4) fiber (2a, 20, 21; Lemay et al., Virology, in press). DOC dissociates complete penton into fiber and penton base (2).*

tions. The fiber-defective mutant *ts*115 underwent assembly until the stage of light IM particles (or top components), which accumulated at 39.5°C, whereas *ts*116 and *ts*125 produced few virus particles at nonpermissive temperatures. Both *ts*116 and *ts*125 synthesized fiber which was abnormal in antigenic properties and polypeptide structure (molecular weight of 60,000 instead of 62,000 for the WT fiber polypeptide unit). Despite the altered fiber structure, *ts*116 and *ts*125 IM particles were capable of maturation upon a shift down to 33°C, resulting in the production of infectious virions with a 60,000-molecular-weight protein in place of the 62,000-molecular-weight polypeptide IV (Fig. 8 and 9). The mutation of *ts*116 and *ts*125 was also expressed at 33°C, and the same 60,000-molecular-

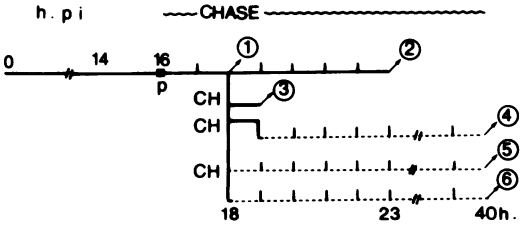

FIG. 6. *Scheme of pulse-chase and shift-down experiments in the presence or absence of CH. KB cells were infected with ts104 or ts125 at 39.5°C, pulse-labeled (P) with [^{35}S]methionine (50 μCi/ml) for 20 min at 16 h after infection (p.i.), and chased at 39.5°C (solid lines) or at 33°C (dotted lines). Samples were withdrawn at different times of the chase (numbers within circles) and analyzed in sucrose gradients to separate nuclear 750S virions and 600S IM particles.*

weight protein was synthesized in infected cells at 33°C (Fig. 2).

*ts*116 and *ts*125, as well as the other fiber- and fiber and penton base-defective *ts* mutants studied here, had an apparently normal glycosylated fiber (Fig. 3). These results suggest that (i) the site of glycosylation of fiber polypeptide corresponds to a region of the virus genome at which it is difficult to induce mutations or (ii) there are several sites of glycosylation. The occurrence in *ts*116 and *ts*125 of polypeptide IV smaller in size by a molecular weight of 2,000 than the WT polypeptide IV led to the following hypotheses: (i) the *ts*116 or *ts*125 or both mutations can generate a new initiation or termination codon on the fiber mRNA, with a polypeptide chain shortened at its N or C end; (ii) the mutations might have created a new preferential cleavage site for a cellular or virus-coded endopeptidase; (iii) whichever end of the *ts*116 or *ts*125 fiber polypeptide chain is modified, the production of infectious *ts*116 and *ts*125 at 33°C with normal infectivity and normal morphology suggests that the first 20 or the last 20 amino acids of the polypeptide IV chain were not indispensable for assembly of the normal fiber trimer structure (29) or for penton base and fiber assembly, at least at 33°C.

TABLE 4. *Pulse-chase analysis of assembly IM and virus particles of HAd2 ts104 with or without a shift down*

Sample	cpm[a]			
	600S IM (A)	750S virus (B)	Total radioactivity (A + B)	Ratio of (B − A)/(A + B)
1	35,900	22,700	58,600	−0.225
2	29,200	15,800	45,000	−0.298
3[b]	20,100	18,400	38,500	−0.044
4[b]	17,700	19,600	37,300	+0.050
5[b]	9,000	15,700	24,700	+0.271
6	12,100	20,000	32,100	+0.246

[a] The numbers correspond to the radioactivity of the samples defined in the legend to Fig. 6.
[b] CH added during the chase.

*ts*104, which is phenotypically fiber and penton base defective, accumulated equivalent quantities of top components and YV at nonpermissive temperatures. In the presence of CH, which has been shown to inhibit the de novo formation of IM particles from the pool of soluble components (28), the YV evolved into mature infectious virions after a shift down to 33°C, as evidenced by the processing of PVI, PVIII, and PVII. This mutant resembled, therefore, the HAd2 *ts*1 mutant of Weber, which is blocked at 39.5°C at a stage of immature virions with un-

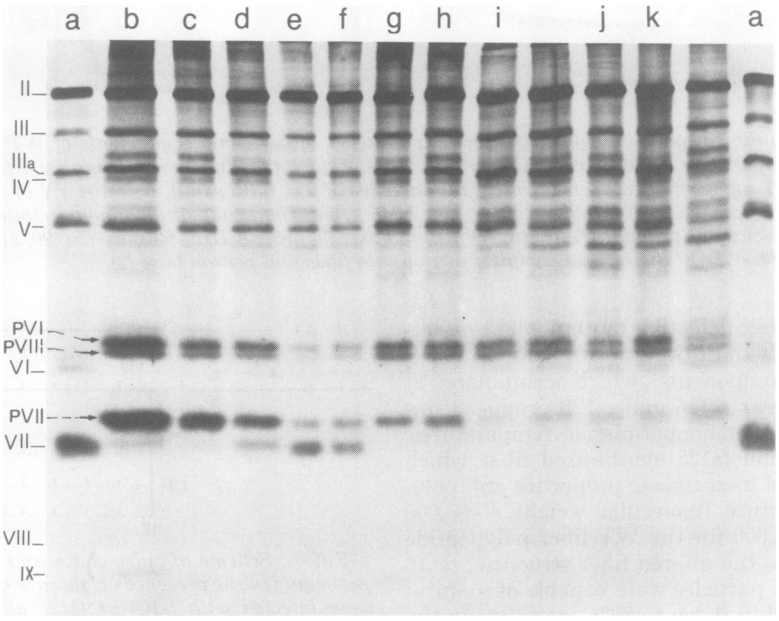

FIG. 7. *Polypeptide pattern of ts104 nuclear 750S YV (b, c, d, e, and f) and 600S IM particles (g, h, i, j, and k) produced during pulse-chase and shift-down experiments, as schematized in Fig. 6. (a) Control HAd2 virion; (b and g) sample 1 of Fig. 6; (c and h) sample 3; (d and i) sample 4; (e and j) sample 6; (f and k) sample 5. The maturation of YV is evidenced by the processing of PVII into VII upon a shift down (d, e, and f), even in the presence of CH (d and f).*

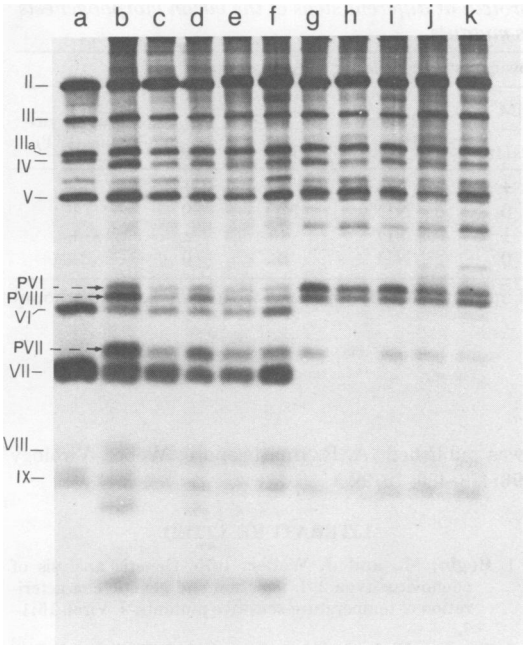

FIG. 8. *Polypeptide pattern of ts125 nuclear 750S virions (b, c, d, e, and f) and 600S IM particles (g, h, i, j, and k) produced during pulse-chase and shift-down experiments, as schematized in Fig. 6. (a) Control HAd2 virion; (b and g) sample 1 of Fig. 6; (c and h) sample 2; (d and i) sample 3; (e and j) sample 5; (f and k) sample 6. Note that after a 7-h chase at 39.5°C, the polypeptide pattern of ts125 750S virus particles resembles that of a WT, with visible maturation cleavage of PVI, PVIII, and PVII (c).*

cleaved precursors (33). These results with *ts*104 further support the hypothesis of a virus-coded or virus-induced cellular endopeptidase responsible for the cleavage maturation of precursors to virion proteins VI, VIII, and VII (30, 31; see Addendum in Proof). In another study, data which suggest a similar maturation processing of polypeptide IIIa in YV were reported (M. L. Boudin, J. C. D'Halluin, C. Cousin, and P. Boulanger, Virology, in press). *ts*104 YV were found to be devoid of a DOC core, confirming the low affinity of precursor PVII for viral DNA at low ionic strength, as previously observed (10, 23).

The antigenic properties of the major capsid subunits incorporated into the viral capsid can be studied qualitatively and quantitatively by two-dimensional immunoelectrophoresis of DOC-disrupted particles (2, 20, 21; Boudin et al., Virology, in press). This technique was applied to the virus antigens present in the different classes of particles accumulated by the *ts* mutants at 39.5°C. In light IM particles (or top components; ρ = 1.30 to 1.31 g/ml), such as those accumulated by *ts*101, *ts*104, *ts*112, and *ts*120, and the ρ = 1.315 g/ml IM particles of the WT (3), only hexon and fiber antigens formed an immune precipitate peak in two-dimensional immunoelectrophoretic analysis (Fig. 5). In DOC-disrupted YV, those produced at 39.5°C by *ts*104, only hexon and fiber antigens were detectable (Fig. 5). In contrast, hexon, penton base, fiber, and IIIa antigens were found in DOC-treated WT mature virions, as well as in *ts*104 mature virions obtained after a shift down. Penton base appeared antigenically active in the ρ = 1.345 g/ml virions produced in minute amounts by *ts*125 at 39.5°C (Fig. 5).

These data suggested a modification of the virus antigens within the capsid during virion morphogenesis, with an eclipse phase affecting the antigenicity of the vertex structures (9). Only hexon antigen seemed to remain constant in antigenicity throughout the assembly pathway (Table 5). The absence of a detectable antigen in two-dimensional immunoelectrophoretic analysis does not necessarily signify a total absence of antigenic sites. As reported elsewhere (Boudin et al., Virology, in press), modified virus

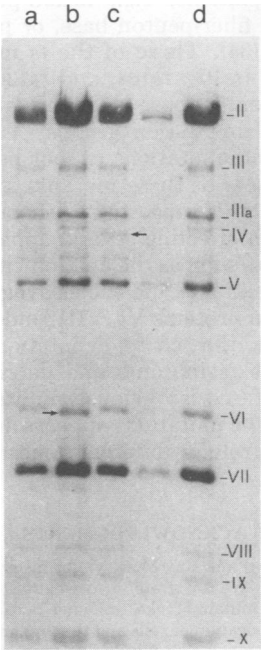

FIG. 9. *Polypeptide pattern of HAd2 infectious virus particles (ρ = 1.345 g/ml) produced at 33°C by (a) ts103, (b) ts115, (c) ts125, and (d) the WT. The molecular weights of polypeptide IV present in ts125 virion (c) and of polypeptide VI present in ts115 virion (b) are lower than those of the corresponding polypeptides of the WT (arrows). Staining: Coomassie brilliant blue R-250.*

TABLE 5. *Antigenic properties of HAd2 major capsid protein at different steps of the virion morphogenesis in WT and ts mutants*

Component	Antigenic properties[a] of following virus particles or subviral entities:								
	Soluble components			1.315-g/ml IM[b]		1.37-g/ml IM[b] (WT)	YV		Mature virions (WT)
	WT	*ts*136	*ts*107	WT	*ts*112		WT	*ts*104	
Hexon	+	+	+	+	+	+	+	+	+
Penton base	+	0	+	0	0	ND[c]	ND	0	+
Fiber	+	0	+	+	+	ND	+	±	+
IIIa[d]	+	0	+	0	0	ND	0	0	+

[a] +, Same immune precipitate peak in two-dimensional immunoelectrophoresis as with WT; ±, trace amount of antigen; 0, no antigen detectable.
[b] Data from references 3 and 4.
[c] ND, Not determined.
[d] Data from Boudin et al., Virology, in press.

antigens retained enough antigenic determinants to be selected on *Staphylococcus aureus* protein A through their specific antibody (15), but not enough to form an immune precipitate lattice within the agarose gel.

Serological and SDS-polyacrylamide gel electrophoretic analyses have shown that a number of *ts* mutants of HAd12 (27), HAd31 (32), and HAd2 (34) fail to synthesize pairs or groups of capsid components, e.g., hexon-penton base, hexon-fiber, fiber-penton base, or polypeptides IIIa-V-80K (34). Three of the *ts* mutants presented here (*ts*103, *ts*104, and *ts*136) appeared fiber and penton base defective and fell into three complementation groups (Table 1). Although double mutations cannot be totally excluded in some of these mutants, the data obtained with *ts*104 suggested a lesion of a regulatory function affecting several apparently nonlinked events, such as the antigenicity of penton base, fiber, and IIIa and the cleavage of precursors to virion proteins VI, VIII, and VII. As the accuracy of the complementation tests for grouping the *ts* mutants and determining the functions altered is obviously limited, physical mapping of the mutations appears necessary for defining the role of different genes in virus assembly.

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ADDENDUM IN PROOF

After completion of this study, a paper describing the partial characterization of an ad2-induced protease was published (A. R. Bhatti and J. Weber, Virology **96**:478–485, 1979).

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