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Temperature-Sensitive Mutant of Adenovirus Type 2 Blocked in Virion Assembly: Accumulation of Light Intermediate Particles

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A temperature-sensitive mutant of human adenovirus type 2, *ts*112, was isolated and characterized. *ts*112 was blocked in a late function required for virus maturation. At restrictive temperature, it accumulated light precursor particles that were able to mature into infectious virions upon temperature shift-down. Use of a mild extraction procedure and a reversible fixation by a cleavable diimido ester permitted the isolation and analysis of these labile intermediates in the adenovirus assembly. These accumulated particles had a sedimentation coefficient of about 600S and a buoyant density of 1.315 g/cm^3 in CsCl. They contained a DNA fragment of 7-11S and two nonvirion proteins having molecular weights of 50,000 (50K) and 39,000 (39K), respectively. They resembled in composition and morphology the light intermediate particles found in wild-type adenovirus 2, which were identified as precursors of heavy intermediates, preceding the young virions. The *ts*112 lesion was apparently located at the exit of either the 50K and/or 39K proteins and at the entry of viral DNA.

The assembly of a virus constitutes a model system for studies on protein-protein and protein-nucleic acid interactions. It also offers a possible clue for the therapy of viral diseases (6, 12). Adenovirion assembly has been studied by different approaches: in vitro reconstruction (4, 31, 32); analysis of incomplete particles, precursors of mature virions (5, 11, 14, 19, 20, 23, 24, 30); and analysis of temperature-sensitive (*ts*) mutants blocked in different steps of viral morphogenesis (28).

In our work, the assembly of human type 2 adenovirus (Ad2) was studied by biochemical and electron microscopic analysis of different classes of particles obtained with a *ts* mutant, *ts*112. These particles were isolated by centrifugation in sucrose and in CsCl gradients after reversible fixation by a cleavable diimido ester (9). *ts*112 was defective in a late stage required for virus maturation. At the nonpermissive temperature, an accumulation of precursor particles, able to mature into infectious virions upon temperature shift-down, was observed.

These light intermediate (IM) particles were reminiscent of the prohead structure described in certain double-stranded DNA bacteriophages (2, 15), and they were also found in wild-type (WT) Ad2, provided that a mild procedure of isolation and fixation was used (9).

MATERIALS AND METHODS

Cells. KB cells were grown in suspension in Joklik-modified medium F 13 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 5% horse serum. HeLa cells were grown as monolayers in Eagle minimum essential medium, containing 10% calf serum.

Virus. The Ad2 WT was originally supplied by J. F. Williams (Carnegie-Mellon Institute, Pittsburgh, Pa.). The Ad2 *ts* mutant, *ts*112, was isolated after nitrous acid treatment of the WT stock. This was done according to the method described by Williams et al. (29), and preliminary characterization has also been performed (G. R. Martin, R. Warocquier, J. C. D'Halluin, and P. A. Boulanger, manuscript in preparation).

Virus production and purification. Stocks of WT and of *ts*112 were grown by infecting cells at a low multiplicity of infection and incubating them at 37°C for 40 h and at 33°C for 96 h, respectively. Virus particles were purified as previously described (3, 9). Infectivity was assayed by the fluorescent focus assay technique (18) or by the plaque method on HeLa cells. The titers were expressed in fluorescent focus units or PFU.

Infection and labeling conditions. Cells were infected at a multiplicity of infection of 25 PFU per cell. KB cells in suspension were centrifuged at low speed at different times postinfection (p.i.) and resuspended at a density of 10^6 cells per ml in culture medium. For pulse-labeling, the cells were labeled with

1 μCi of [^{14}C]valine per ml and 2 μCi of [^{3}H]thymidine per ml in a valine-deprived medium. For chase experiments, the cells were diluted in normal medium at 3 $\times$ 10^5 cells per ml. HeLa-cell monolayers were pulse-labeled with 6 μCi of [^{35}S]methionine per ml in a medium containing 2.5% of the normal methionine concentration. For *ts*112, the permissive temperature was 33°C and the restrictive temperature was 39.5°C.

Radioisotopes. [^{35}S]methionine (600 to 700 Ci/mmol) and [^{14}C]valine (250 to 300 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England), and [^{3}H]thymidine (30 Ci/mmol) was purchased from the Commissariat à l'Energie Atomique (Saclay, France).

Cell fractionation and isolation of virus and IM particles. Cell fractionation was performed according to a modification of the technique of Edvardsson et al. (11). Infected cells were washed with cold phosphate-buffered saline, suspended at 4 $\times$ 10^7 cells per ml in reticulocyte standard buffer (10 mM Tris-hydrochloride, pH 7.4-10 mM NaCl-1.5 mM $MgCl_2$), and left for 10 min at 0°C. Triton X-100 was added up to a final concentration of 0.5%, and the cells were disrupted by 10 strokes in a tight-fitting Dounce homogenizer. NaCl was added up to 100 mM, and the cell lysate was centrifuged at 1,000 $\times$ *g* for 5 min. The supernatant, referred to as the cytoplasmic fraction, was adjusted to 20 mM sodium EDTA, centrifuged at 16,000 $\times$ *g* for 10 min, and analyzed on a sucrose or Ficoll gradient, as described below. The nuclei in the pellet was lysed by the method of Wallace and Kates (26). They were suspended in 50 mM Tris-hydrochloride, pH 8.0-10 mM sodium EDTA (TE buffer) adjusted to 0.3 M $(NH_4)_2SO_4$, homogenized in a tight-fitting Dounce homogenizer (three strokes), and immediately diluted with 2 volumes of TE buffer. The nuclear lysate was then centrifuged on a 30% (wt/vol) sucrose cushion at 16,000 $\times$ *g* for 10 min. The supernatant was further analyzed on sucrose (E. Merck A. G., Darmstadt, West Germany) or Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradients. The gradients, containing 5 to 12.5% Ficoll in 50 mM Tris-hydrochloride, pH 8.0-150 mM NaCl-10 mM sodium EDTA or 25 to 40% sucrose in 50 mM Tris-hydrochloride, pH 8.0-200 mM NaCl-10 mM sodium EDTA, were centrifuged at 85,000 $\times$ *g* and 4°C for 105 min in an SW 27 rotor over a cushion of CsCl (1.43 g/cm^3). The gradients were collected dropwise from the bottoms of the tubes, and the fractions were assayed for acid-precipitable radioactivity on Whatman GF-C filters.

Fixation of virus particles. For electron microscopic analysis, a nonreversible fixation of samples was carried out with 5% glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.0) at room temperature for 30 min. Excess fixation by glutaraldehyde was prevented by adding 0.1 M lysine, pH 7.4, for an extra 2 h at room temperature, followed by dialysis against phosphate-buffered saline.

For biochemical analysis, a reversible fixation by a cleavable diimido ester was used (25). This second procedure was performed on virus particles obtained from sucrose or Ficoll gradients made in 20 mM sodium borate, pH 8.0, instead of 50 mM Tris-hydrochloride, pH 8.0. Samples were first reacted with 3 mg of methyl-4-mercaptobutyrimidate hydrochloride (Pierce Chemicals Co., Rockford, Ill.) per ml for 30 min at 4°C. Excess reagent was removed by dialysis against phosphate-buffered saline, containing 100 mM H_2O_2 to provoke disulfide bridge formation. The induced disulfide cross-bridges were cleaved by 2-mercaptoethanol to perform polypeptide analysis on sodium dodecyl sulfate (SDS)-acrylamide gels.

Analytical SDS-polyacrylamide gel electrophoresis. The samples were dissolved in SDS denaturing mix (62.5 mM Tris-hydrochloride, pH 6.8-4% SDS-10% 2-mercaptoethanol-6 M urea) and heated for 2 min at 100°C. The polypeptides were analyzed on a 17.5% acrylamide-0.08% bisacrylamide slab gel overlayered by a 5% acrylamide-0.13% bisacrylamide stacking gel in the discontinuous buffer system of Laemmli (16). Electrophoresis was carried out for 16 h at 30 V (constant voltage) in a Bio-Rad model 220 apparatus. The gels were stained with Coomassie brillant blue R 250, vacuum-dried, and autoradiographed with Kodak Kodirex film.

Electron microscopy. The different classes of virus particles obtained from sucrose or Ficoll gradients were examined in a Hitachi HU-12 electron microscope after staining with 1% potassium phosphotungstate, pH 7.2. They were examined nonfixed or fixed with glutaraldehyde (as above).

Extraction of DNA from virus particles. DNA present in mature virions or in IM particles was analyzed according to the procedure described by Doerfler (10), with the following modification: 10% 2-mercaptoethanol was added to the lysis buffer (0.5 M NaOH-50 mM sodium EDTA) to cleave the disulfide cross-links.

RESULTS

Comparison of virus-coded proteins in *ts*112- and WT-infected cells by pulse-chase experiments. Preliminary phenotypic characterization of the *ts* mutants of Ad2 has shown that *ts*112 induced normal synthesis of viral DNA and the major soluble antigens at the restrictive temperature. Moreover, electron microscopic controls have revealed that the *ts*112-infected cells maintained at 39.5°C contained a great number of particles with cores less dense than those of mature virions. It seemed, therefore, likely that the *ts*112 lesion was in a late function, such as precursor protein cleavage. It is known that several adenovirus proteins are derived from precursor polypeptides by specific processing (1, 11, 14, 17).

HeLa cells infected with *ts*112 and WT and maintained at 39.5 or 33°C were pulse-labeled with [^{35}S]methionine. Chase was performed in a fresh medium containing a fivefold excess of cold methionine, and incubation was continued for various lengths of time at 39.5 or 33°C. The cells were harvested, and labeled polypeptides were analyzed on SDS-polyacrylamide gels. The patterns of pulse-labeled polypeptides in *ts*112- and

WT-infected cell extracts were almost identical at 39.5°C (Fig. 1). However, significant differences could be observed in the chase experiments. The precursor polypeptide P VI (27,000 daltons [27K]) seemed to be processed, and a weak band of VI (24K) appeared. In contrast, the precursor P VII scarcely diminished, and its cleavage product, virion polypeptide VII, failed to appear during the chase in *ts*112-infected cells. The absence of cleavage P VII → VII, which has been shown to be a late event (1, 27, 28), therefore suggested that *ts*112 was defective in virus particle maturation. No structural or nonstructural viral polypeptides appeared to be thermosensitive at 39.5°C.

Isolation of *ts*112 particles by density centrifugation in CsCl. Since it appeared that *ts*112 was blocked at a late step in viral assembly, it was interesting to determine which classes of virus particles were produced in infected cells incubated at either the restrictive (39.5°C) or the permissive (33°C) temperature. *ts*112 was

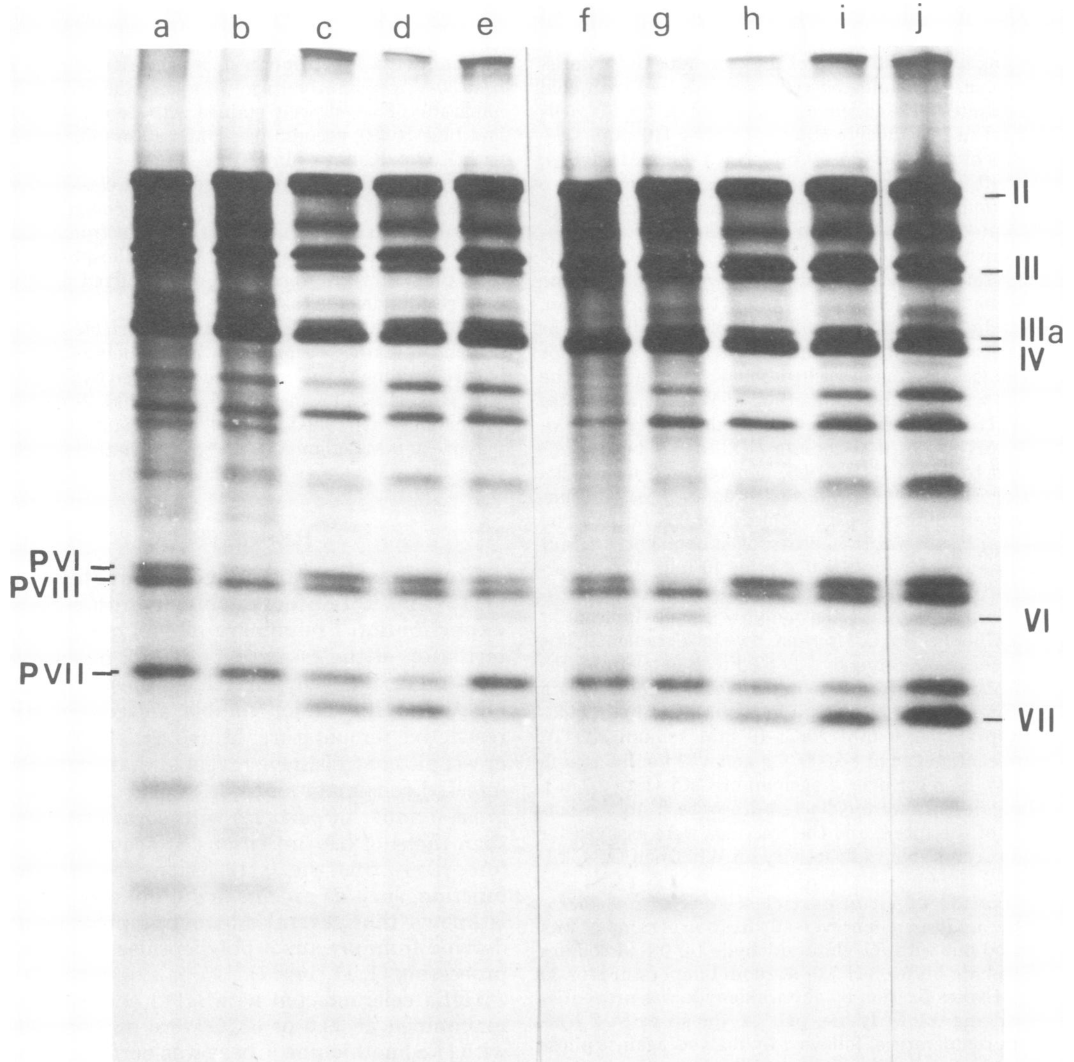

FIG. 1. *Autoradiogram of SDS-polyacrylamide gel of WT- and ts112-induced polypeptides in HeLa cells. HeLa cell monolayers infected with ts112 (a–e) and WT (f–j) were pulse-labeled with [^{35}S]methionine for 1 h at 23 h p.i. and 39.5°C and for 3 h at 69 h p.i. and 33°C and chased for different lengths of time at either 39.5 or 33°C. (a, f) Pulse at 39.5°C; (b, g) pulse and chase at 39.5°C for 16 h; (c, h) pulse at 33°C; (d, i) pulse and chase at 33°C for 24 h; (e, j) pulse at 33°C, shift-up after pulse, and chase at 39.5°C for 24 h. The cleavage of P VII into VII appears altered in ts112-infected cells at 39.5°C. Anode is at the bottom.*

grown at 39.5 and 33°C in KB cells for 36 and 80 h, respectively. The particles were extracted as described in Materials and Methods and analyzed on a self-generating CsCl gradient with an initial density of 1.34 g/cm^3. The gradient patterns showed: (i) at 33°C, a major band of virions with a buoyant density of 1.345 g/cm^3 and a minor band in the 1.29- to 1.30-g/cm^3 density zone; (ii) at 39.5°C, a minor band of mature virions and two major bands at densities close to each other, at 1.29 and 1.30 g/cm^3 (not shown). These two latter bands were collected separately and further purified from each other on preformed 1.23- to 1.34-g/cm^3 CsCl gradients.

The different bands were collected, dialyzed against 10 mM Tris-hydrochloride, pH 8.0–1 mM sodium EDTA, and heated in SDS denaturing mix, and then the polypeptides of these particles were analyzed on SDS-polyacrylamide slab gels. The light particles at 33°C (Fig. 2c) and the 1.30-g/cm^3 particles at 39.5°C (Fig. 2f) contained the same species of polypeptides as the empty particles described in the Ad3 system (11). They lacked almost totally the core proteins V and VII and contained instead the polypeptides 27K and 26K, probable precursors of virion polypeptides VI and VIII. There was a relatively greater proportion of 26K. The cleavage products VI and VIII were also present in small amounts. The 1.29-g/cm^3 particles at 39.5°C (Fig. 2g) were similar in polypeptide composition to WT incomplete particles (11, 20), except that these *ts*112 particles contained a small amount of P VII and almost no VIII and that the respective proportions of 27K and 26K were changed in favor of the 27K. The light 1.30-g/cm^3 particles produced at 39.5°C were analyzed with regard to their DNA content and were found to contain a fragment of DNA sedimenting in an alkaline sucrose gradient as a broad peak between 7 and 11S (Fig. 3). No detectable DNA was found in 1.29-g/cm^3 particles at 39.5°C. The presence of significant amounts of precursor polypeptide P VII, besides the major band of core protein VII, in the 1.345-g/cm^3 particles produced at 33°C (Fig. 2b) suggested that these particles were in some way an intermediate state between young (14) and mature virions. These results suggested that *ts*112 was blocked in a step preceding the formation of young virions. The occurrence of a large amount of light particles, even at 33°C, implied that the mutation was still expressed at the permissive temperature.

Isolation of the different classes of *ts*112 particles on sucrose or Ficoll gradients. The preceding results established that *ts*112 grown at 39.5°C produced light particles banding at a density of 1.29 to 1.30 g/cm^3 in a CsCl gradient.

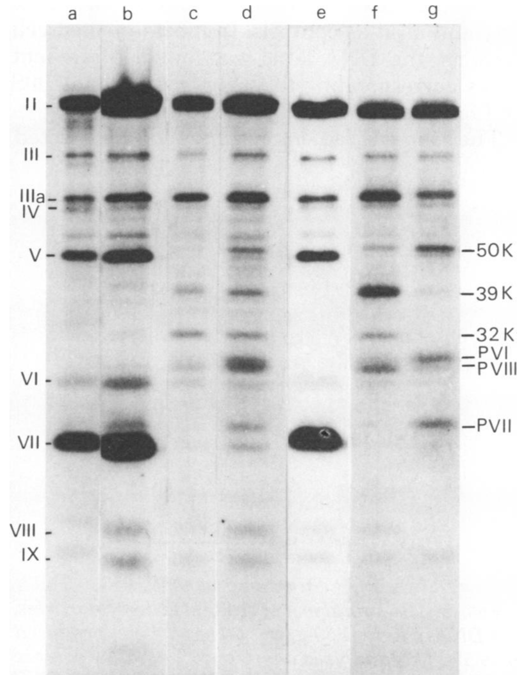

FIG. 2. *Polypeptide composition of Ad2 ts112 particles. Particles produced by ts112 at the permissive or the restrictive temperature were extracted from infected cells at 36 h p.i. at 39.5°C and at 80 h p.i. at 33°C and isolated on CsCl gradients without prior fixation. (a) Ad2 WT; (b) ts112 particles produced at 33°C and banding at 1.345 g/cm^3 in CsCl; (c) light ts112 particles produced at 33°C and found at 1.29 to 1.30 g/cm^3; (d) light ts112 particles produced at 39.5°C (1.29 to 1.30 g/cm^3). The light ts112 particles produced at 39.5°C consisted of two populations, which were further repurified on preformed CsCl gradients. (e) Control WT; (f) 1.30-g/cm^3 ts112 particles; (g) 1.29-g/cm^3 ts112 particles. (a–d) and (e–g) were two separate runs. Staining: Coomassie brilliant blue. Anode is at the bottom.*

Since it has been recently shown that adenovirus assembly IM are fragile structures, easily disrupted by purification in a CsCl gradient without fixation, the accumulated particles of *ts*112 were isolated by a mild procedure recommended by Edvardsson et al. (11). Suspension cultures of KB cells infected with *ts*112 were incubated at 39.5°C and double-labeled with [^{3}H]thymidine and [^{14}C]valine from 12 to 20 h p.i. The cells were fractionated into cytoplasm and nuclei. Extracts from each fraction (2 × 10^8 cells) were layered on top of sucrose or Ficoll gradients. Although Ficoll has been shown to be less damaging to adenovirus IM than is sucrose (11), our results showed that sucrose and Ficoll have the same effect on *ts*112 light particles. Double-labeled, mock-infected cell extracts and WT-in-

fected cell extracts were analyzed in the same way and used as controls. In mock-infected cell extracts, the DNA label was found in gradient zones corresponding to values lower than 100S and larger than 1,000S (not shown).

The sucrose gradient pattern of *ts*112-infected nucleus extract showed a slow-sedimenting, sharp peak at about 600S, referred to as *ts*112 IM; these IM contained little DNA label (Fig. 4b). The extracts from WT-infected nuclei contained two peaks. One sharp peak sedimented as intact virions at 750S and was labeled in both the DNA and the protein moieties. Another broad peak, slower than 750S, contained little DNA and had a heterogeneous sedimentation rate (Fig. 4a). The analysis of these extracts on Ficoll gradients showed the same patterns (not shown). The fractions corresponding to each peak were pooled, and the virus particles were fixed with glutaraldehyde for electron microscopic analysis and with cleavable diimido ester for further biochemical analysis, i.e., density determination in a CsCl gradient and polypeptide analysis on an SDS-polyacrylamide gel.

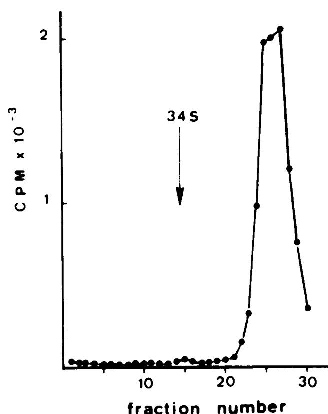

FIG. 3. *DNA analysis of Ad2 ts112 light particles. The DNA of light, 1.30-g/cm³ ts112 particles produced at 39.5°C was analyzed on a 5 to 20% alkaline sucrose gradient. The position of WT DNA (34S) is indicated by the arrow. The DNA of light ts112 particles behaves as a heterogeneous population of molecules with sedimentation coefficients ranging from 7 to 11S. Bottom is at the left.*

Density analysis of the *ts*112 IM particles. Fixed *ts*112 IM particles were analyzed in a CsCl gradient. A single sharp peak was obtained at a density of 1.315 g/cm³ (Fig. 5). The sharpness of this peak suggested a strong homogeneity in the population of these IM particles. The value of 1.315 g/cm³ and, thus, the ratio of DNA to protein content were higher than those previously determined for empty and incomplete particles (8, 11, 20). This suggested that the mild extraction procedure followed by fixation preserved the structure of the *ts*112 IM.

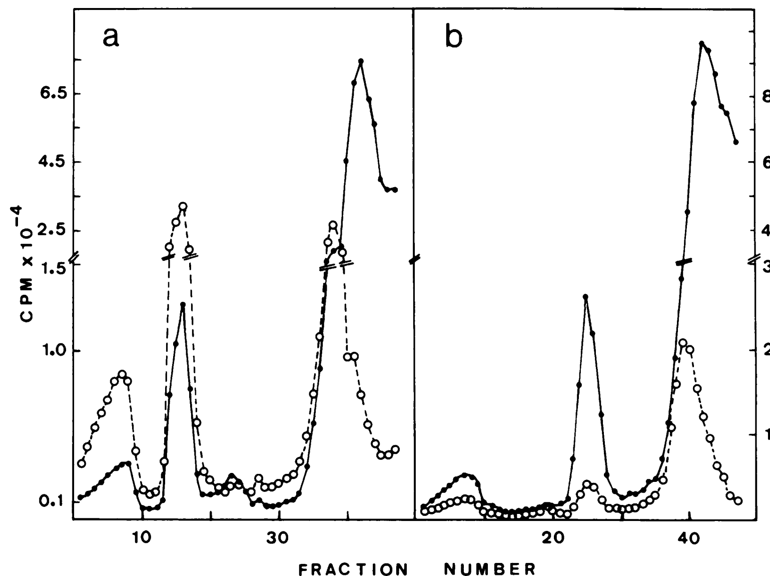

FIG. 4. *Sucrose gradient centrifugation analysis of nuclear extracts from WT- and ts112-infected KB cells. Ad2 WT- or ts112-infected KB cells were labeled with [³H]thymidine and [¹⁴C]valine from 12 to 20 h p.i., and nuclear extracts were analyzed on 25 to 40% sucrose gradients. (a) WT; (b) ts112. The peak at fraction 16 in (a) corresponds to adenovirus particles with an apparent sedimentation coefficient of 750S. The peak at fraction 25 in (b) corresponds to ts112 accumulated IM sedimenting at 600S. Symbols: ●, ¹⁴C; ○, ³H. Bottom is at the left.*

Electron microscopy of the *ts*112 IM particles. The *ts*112 material, isolated on CsCl gradient without prior fixation and banding at 1.29 to 1.30 g/cm^3, was found to contain empty particles, penetrated by stain, along with disrupted capsids, confirming the lability to adenovirus intermediates in CsCl (not shown).

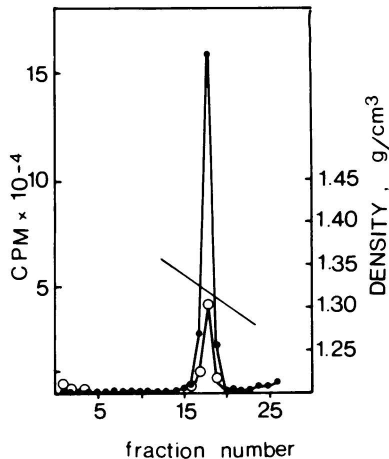

FIG. 5. *Isopycnic gradient centrifugation of ts112 IM. The ts112 IM particles, isolated on a sucrose gradient as in Fig. 4, were fixed with diimido ester and further analyzed in a CsCl gradient. A sharp band sedimenting at 1.315 g/cm^3 was obtained. Bottom is at the left.*

In the *ts*112 material isolated on a sucrose gradient and nonfixed, a unique class of particles was observed (Fig. 6a). These particles appeared similar in size and contour to mature virions, but gaps were visible in the outer capsid and the icosahedral shape was not discernible. The same particles, after fixation with glutaraldehyde (Fig. 6b), appeared rounder than mature virions and the stain penetrated irregularly into the capsids.

Polypeptide pattern of the *ts*112 IM particles. Diimido ester-fixed *ts*112 IM particles, obtained from Ficoll or sucrose gradients and further purified in CsCl gradients, were dissolved in SDS denaturing mix, and their polypeptide components were analyzed on SDS-polyacrylamide gels. No significant difference was observed between the polypeptide patterns of nonfixed IM particles obtained from Ficoll or sucrose gradients and those of fixed IM particles obtained by either method after subsequent purification in a CsCl gradient, except for the disappearance in the fixed particles of a barely visible band of P VII (Fig. 7). This suggested an effective fixation by the cleavable diimido ester. The P VII present in the IM peak of the sucrose gradient was also found in all the gradient fractions, and, most likely, it represented contaminating material that was subsequently elimi-

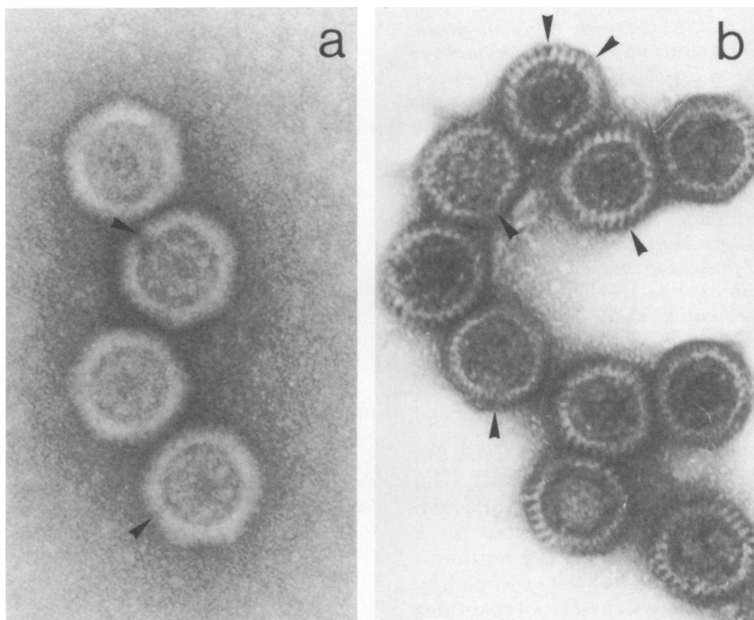

FIG. 6. *Electron microscopy of ts112 IM particles. The ts112 600S particles, obtained from a sucrose gradient (as on Fig. 4), were examined nonfixed (a). The same ts112 particles were fixed with glutaraldehyde and further purified in a CsCl gradient, and the population banding at 1.315 g/cm^3 was examined in the same conditions (b). Staining: potassium phosphotungstate. The arrows indicate gaps in the virus capsids.* ×240,000.

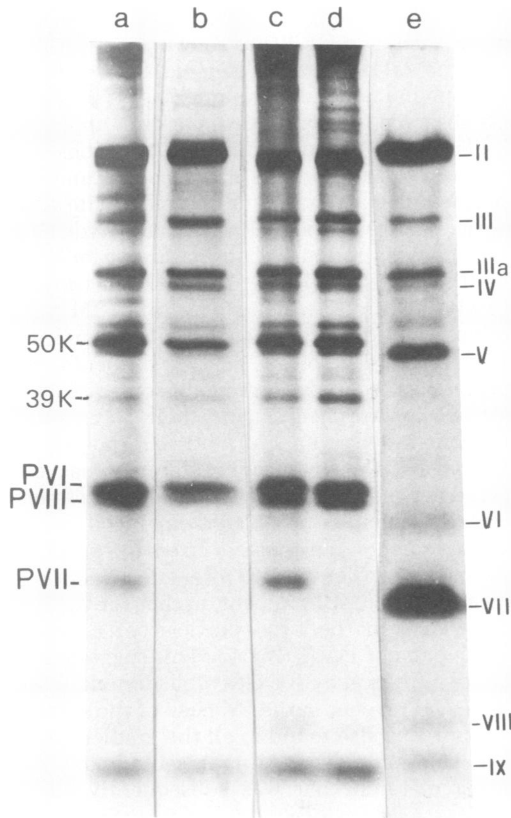

FIG. 7. *Polypeptide composition of ts112 IM particles extracted and isolated with different procedures. (a) ts112 IM extracted by sonic treatment of nuclei and isolated on Ficoll gradient before fixation, as described by Edvardsson et al. (11); (b) the same material after diimido ester fixation and further purification in a CsCl gradient; (c) ts112 IM particles extracted by ammonium sulfate treatment of nuclei and isolated on a sucrose gradient before fixation; (d) the same material after diimido ester fixation and banding in a CsCl gradient; (e) control WT. Traces of precursor P VII present in material cosedimenting with IM in a Ficoll (a) or sucrose (c) gradient disappeared upon repurification of the fixed material by banding in CsCl (b, d). The fixed purified particles in (b) and (d) show similar polypeptide patterns. Anode is at the bottom.*

nated by fixation and purification in CsCl (9).

The virion structural polypeptides II, III, IIIa, IV, IVa_2, and IX were present in the IM particles. Polypeptide V was barely visible on a stained gel and on the corresponding autoradiogram. Polypeptides VI and VII were completely missing. In contrast, the precursor polypeptides P VI and P VIII were strongly labeled. Two extra polypeptides, with apparent molecular weights of 50,000 and 39,000, were found in great amounts. This polypeptide pattern differed significantly from that obtained with CsCl-isolated light particles (Fig. 2), confirming the lability of nonfixed adenovirus IM in high-ionic-strength medium (11). The light particles obtained in CsCl gradients without prior fixation probably corresponded to breakdown products of assembly IM.

Reversibility of the mutation by temperature shift-down. The reversibility time of the mutation was determined by titrating the infectious *ts*112 obtained after shift-down at different times p.i. Temperature-shifted cultures were harvested at 87 h p.i., and infectious virus was titrated by fluorescent focus assay on crude cell lysate. Figure 8 compares the yields of infectious *ts*112 obtained after shift-down with the growth curves at each temperature. Until 18 h p.i., there was little or no effect of the nonpermissive temperature on the final virus yield. From 30 to 50 h, the effect of the mutation could not be reversed by a shift-down to a permissive temperature. Therefore, the critical period for the expression of the lesion seemed to extend from 18 to 30 h p.i. at 39.5°C. These data were used in the next experiment to choose the proper periods of labeling and temperature change.

Evolution of *ts*112 IM particles upon tem-

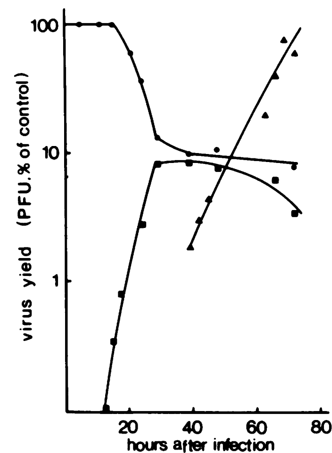

FIG. 8. *Temperature dependence of ts112 infectious particle maturation. Duplicate sets of ts112-infected KB-cell cultures were incubated at 33 or 39.5°C for different lengths of time and then harvested for determination of the virus yield at each temperature. One 39.5°C culture was shifted down to 33°C and incubated at this temperature until 87 h p.i. The virus production was titrated by the fluorescent focus method, and the results are expressed as a percentage of the virus yield at 87 h p.i. at 33°C. The x axis indicates the time p.i. at which cultures were harvested or shifted down. Symbols: ▲, ts112 growth curve at 33°C; ■, ts112 growth curve at 39.5°C; ●, ts112 yield after the shift-down to 33°C and further incubation until 87 h p.i. at 33°C.*

perature shift-down. Temperature shift-down experiments after pulse-labeling were performed to determine whether the *ts*112 IM particles could evolve into mature virions or other types of precursors. The length of the chase at 39.5°C, preceding the temperature shift-down, was first determined to get a maximum entry of the label into the IM particles and to test the efficiency of maturation of the virions. KB cells infected with *ts*112 at 39.5°C were labeled with [^{14}C]valine from 15 to 17 h p.i. and then shifted down to 33°C at 17, 20, and 24 h p.i., i.e., after 0-, 3-, and 7-h chase periods. The incubation at 33°C was continued until 88, 80, and 72 h p.i., respectively. The particles produced at the ends of these incubation periods were extracted with Freon and analyzed on CsCl gradients. A 7-h chase period at 39.5°C, preceding the shift-down, resulted in low recovery of label in mature virions (Fig. 9). In contrast, a 3-h chase at 39.5°C, preceding the shift-down, did not affect the entry of label into virions. Since it has been shown that in WT 60 to 90 min is required for appearance of maximum radioactivity in intermediates (14), a chase period of 2 h at 39.5°C, before shift-down, was chosen for the next experiment.

KB cells infected with *ts*112 at 39.5°C and labeled with [^{14}C]valine from 15 to 17 h p.i. were chased for 2 h at 39.5°C and then shifted down to 33°C. Samples of 2 × 10^8 cells were withdrawn at different times. The cells were fractionated, and nuclear and cytoplasmic extracts were analyzed on sucrose gradients. The total radioactivity was constant throughout the chase. Figure 10 shows the distribution of nuclear label in the sucrose gradients at 17 (a), 19 (b), 20 (c), 21 (d),

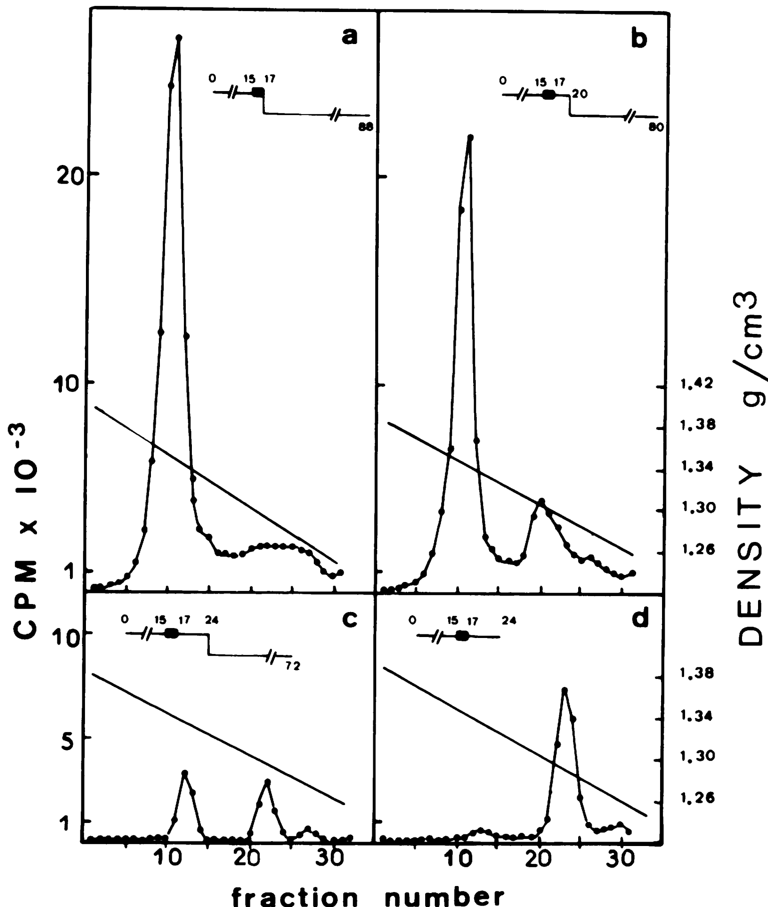

FIG. 9. *Isopycnic centrifugation of ts112 IM matured upon temperature shift-down. ts112-infected KB-cell cultures maintained at 39.5°C were pulse-labeled with [^{14}C]valine for 2 h at 15 h p.i., chased at 39.5°C for various lengths of time, and then shifted down to 33°C. The particles were extracted with Freon at the end of the incubation period and analyzed on CsCl gradients. (a) 0-h chase at 39.5°C; (b) 3-h chase at 39.5°C; (c) 7-h chase at 39.5°C; (d) control 7-h chase at 39.5°C without shift-down. Bottom is at the left.*

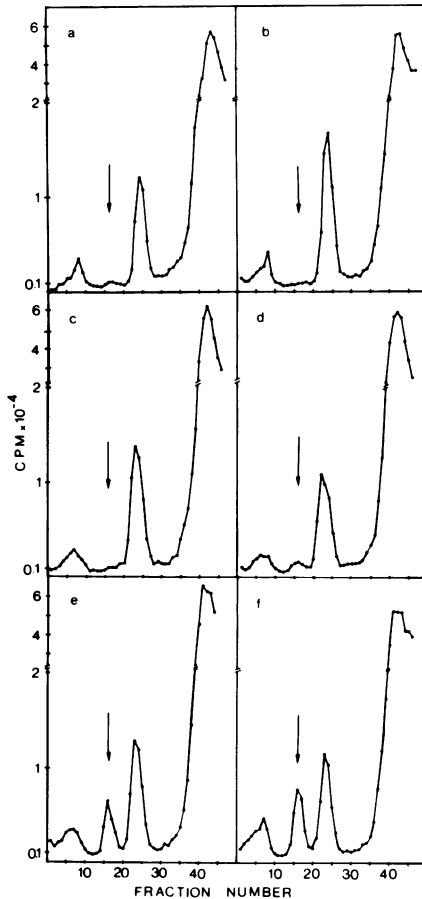

FIG. 10. *Sucrose gradient centrifugation analysis of nuclear ts112 IM matured after shift-down. ts112-infected KB-cell cultures maintained at 39.5°C were labeled with [14C]valine for 2 h at 15 h p.i. (a), chased for 2 h at 39.5°C (b), and then shifted down to 33°C (c–f). Samples were withdrawn at different times, and nuclear extracts were analyzed on 25 to 40% sucrose gradients. (a) Control 2-h pulse at 39.5°C; (b) 2-h pulse at 39.5°C followed by 2-h chase at 39.5°C; (c) 1-h chase at 33°C; (d) 2-h chase at 33°C; (e) 4-h chase at 33°C; (f) 7-h chase at 33°C. Bottom is at the left.*

23 (e), and 26 (f) h p.i. The label in the IM-particle peak increased during a 2-h chase at 39.5°C and then decreased during a chase at 33°C. A peak of nuclear virus appeared at 21 h (2 h after shift-down) and increased at 23 and 26 h p.i. A peak of cytoplasmic virus was detected at 23 h p.i., i.e., 4 h after temperature shift-down (not shown). The ratios of counts per minute in the IM peaks to the total label loaded on the gradients were (in percentages): (a) 6.64; (b) 8.99; (c) 8.74; (d) 6.94; (e) 6.58; and (f) 6.53. Thus, the label in the IM peaks remained almost constant from 4 to 7 h of chase after the shift-down to 33°C.

The fact that the label in the IM peaks remained constant throughout the chase at permissive temperature, whereas the label increased in the virus peak, suggested either a de novo assembly of virions after shift-down or both a maturation of IM into virions and a formation of IM from soluble components at 33°C. However, it has to be considered that the rates of viral maturation are different at 33 and 39.5°C. This rate has been found to be approximately threefold higher at 39.5 than at 33°C (unpublished data). If the time at 33°C is corrected by a factor of 3, the peak of nuclear virus appeared first at 40 min after shift-down, in corrected time. Since it has been shown that in WT the nuclear virus peak is labeled at 1.5 h after pulse (11), it seemed unlikely that the nuclear virus label observed in Fig. 10d originated from a de novo assembly.

Effect of cycloheximide on the evolution of *ts*112 IM particles. Since the appearance of label in the mature virion peak might be due to a de novo assembly from either soluble labeled material or IM-breakdown products, it was of interest to study the fate of the IM and the appearance of mature virions in the presence of cycloheximide (20 μg/ml) as a protein synthesis inhibitor.

A KB-cell culture infected with *ts*112 at 39.5°C was pulse-labeled with [35S]methionine for 15 min at 14.45 h p.i. and divided into 15 aliquots treated as indicated in Fig. 11. Nuclear and cytoplasmic extracts were pooled, to compensate possible nuclear leaks, and analyzed on sucrose gradients. The same amounts of label were loaded on the gradients. The radioactivity increased in the IM fraction until 2 h of chase at 39.5°C and then decreased (Table 1). The amount of label was lower in the IM peak and higher in the virion fraction when the shift-down to 33°C was performed at 17 rather than at 19 h p.i.

Addition of cycloheximide just after the pulse reduced drastically the assembly of IM particles. The same effect has been described for emetine (23). In contrast, the radioactivity in the IM and virus fractions was more important when cycloheximide was added at the time of shift-down, i.e., 2 h after the pulse. Thus, the cycloheximide treatment, performed just after the pulse or 2 h after the pulse, did not prevent the appearance of label in the virions when the culture was shifted down to 33°C.

These results suggested that most of this virus label derived from maturation of the IM particles accumulated at 39.5°C, and not from a de novo assembly of soluble material occurring upon the shift-down to 33°C. In addition, if IM-breakdown products were utilized in virion for-

mation, the efficiencies of reassembly upon shift-down would be similar at 17 and 19 h p.i. This was not the case, as shown in Table 1.

Polypeptide pattern of *ts*112 particles obtained in pulse-chase experiments. The fractions corresponding to the different peaks of the sucrose gradients of Fig. 10 were pooled and treated with the cleavable cross-linking reagent. The different classes of particles were further purified by centrifugation in CsCl gradients and analyzed on SDS-polyacrylamide gels. Figure 12 shows the polypeptide patterns of *ts*112 nuclear IM and virus particles obtained after temperature shift-down. The polypeptide patterns of the *ts*112 IM remained essentially unchanged throughout the chase: a 28K polypeptide visible just after the pulse disappeared during the chase, whereas a 32K polypeptide appeared. Virion polypeptide VII was absent, and traces of precursor P VII were scarcely visible. Polypeptides VI and VIII were present in slight amounts as well as polypeptide V. The nuclear virus consisted mainly of young virions, as suggested by the incomplete processing of P VI and 26K into VI and VIII and of P VII into VII (11, 14). The chase revealed no intermediate pattern between *ts*112 IM (containing the 50K and 39K) and the *ts*112 young virions. However, careful analysis of CsCl gradients of fixed nuclear particles, obtained after 4 and 7 h of shift-down, showed a minor but constant particle population banding at 1.37 g/cm^3. These short-lived 1.37-g/cm^3 intermediates were more easily detected in Ad2 WT (9).

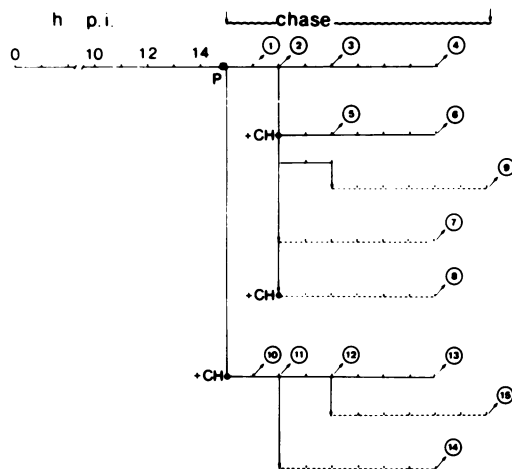

FIG. 11. *Pulse-chase and shift-down experiments in the presence or absence of cycloheximide. KB cells were infected with ts112 at 39.5°C and pulse-labeled (P) with [^{35}S]methionine (20 μCi/ml) for 15 min at 14.45 h p.i. The culture was then divided into 15 aliquots. Solid lines: subculture maintained at 39.5°C; dotted lines: subcultures shifted down to 33°C; CH: cycloheximide. Figures in circles indicate the samples withdrawn after a certain period of chase to analyze the different classes of particles, as in Fig. 10. Quantitative data are given in Table 1.*

DISCUSSION

ts mutant *ts*112 of human Ad2 induced normal synthesis of viral DNA and of viral structural and nonstructural proteins. It also produced more than 1,000 physical particles per cell, for an input multiplicity of 20 to 25 PFU per cell. However, it did not process the precursor protein of major core protein VII, although it cleaved the other precursor proteins, P VI and P VIII. In that respect it seems different from Ad2 *ts*1, which is blocked in the processing of viral protein precursors P VII, P VI, and P VIII (28). Two classes of light particles, banding at 1.29

TABLE 1. *Evolution of label in ts112 intermediates and virions in pulse-chase and shift-down experiments*[a]

Cycloheximide added	Peak[b]	^{35}S incorporation (cpm)					
		Chase period at 39.5°C				Chase periods at 39.5 and 33°C	
		1 h	2 h	4 h	8 h	2 h/39.5 + 6 h/33	4 h/39.5 + 6 h/33
No	IM	16,644 (1)	29,805 (2)	27,991 (3)	22,389 (4)	19,388 (7)	25,910 (9)
	V	0	385	824	854	6,765	4,508
2 h after pulse	IM	16,644 (1)	29,805 (2)	25,189 (5)	24,972 (6)	23,290 (8)	ND[c]
	V	0	385	408	210	9,492	ND
Just after pulse	IM	7,851 (10)	5,890 (11)	5,527 (12)	2,654 (13)	6,560 (14)	4,926 (15)
	V	0	182	53	32	1,572	343

[a] KB cells in suspension were infected with *ts*112 and pulse-labeled at 39.5°C with [^{35}S]methionine (20 μCi/ml) for 15 min at 14.45 h p.i. The culture was divided into 15 aliquots treated as indicated in Fig. 11, and particles were analyzed on sucrose gradients. Values in table are counts per minute found in each particle peak after correction for background. Figures in parentheses refer to the experiment numbers in Fig. 11.

[b] V, Virion peak (750S); IM, IM particle peak (600S).

[c] ND, Not determined.

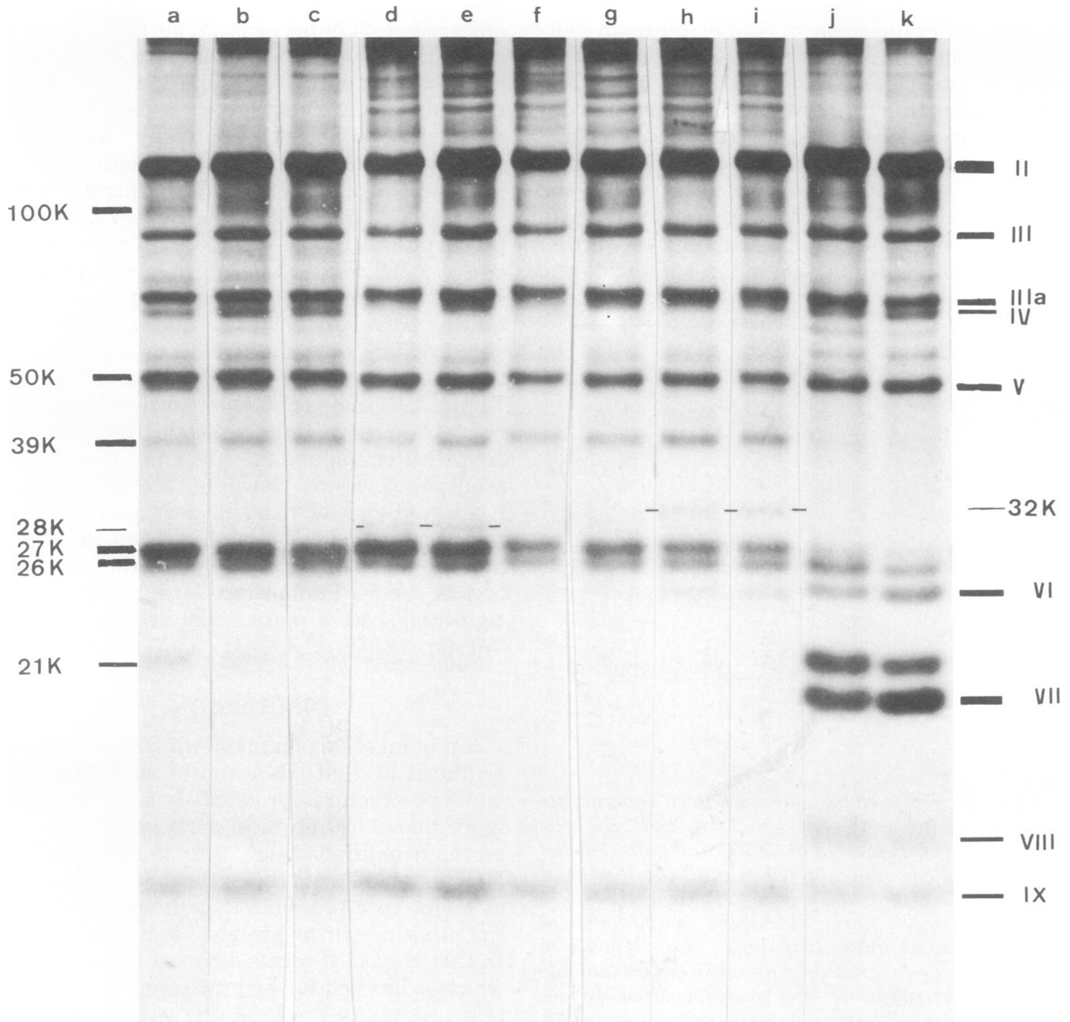

FIG. 12. *Polypeptide pattern of ts112 nuclear IM pulsed and chased at 39.5°C and shifted down to 33°C at various times.The nuclear IM, isolated on sucrose gradients as in Fig. 10, were analyzed before (a–c) or after (d–k) diimido ester fixation and further purification on a CsCl gradient. (a) Nonfixed IM peak (600S) of Fig. 10a; (b) nonfixed IM of Fig. 10b; (c) nonfixed IM of Fig. 10c; (d) fixed IM of Fig. 10a; (e) fixed IM of Fig. 10b; (f) fixed IM of Fig. 10c; (g) fixed IM of Fig. 10d; (h) fixed IM of Fig. 10e; (i) fixed IM of Fig. 10f; (j) fixed virion peak (750S) of the gradient of Fig. 10e; (k) fixed virions of the gradient of Fig. 10f. The polypeptide patterns in (j) and (k) are typical of young virions. As observed in Fig. 7, traces of precursor P VII, present in nonfixed material cosedimenting with IM particles (a–c), disappeared in fixed and repurified material (d–f). Anode is at the bottom.*

and 1.30 g/cm^3 in a CsCl gradient, were produced by *ts*112 at the restrictive temperature. Their polypeptide patterns resembled those of incomplete particles (11, 20, 30) and those of empty particles, or top components (11, 14), found in productive infection with Ad2 WT.

Because CsCl gradient separation has been found to induce the formation of artifactual particles from virus IM (11), a mild method of extraction and a reversible fixation of particles by a cleavable diimido ester were performed before further analysis (9). Using this procedure, it was possible to isolate, on a Ficoll or sucrose gradient, *ts*112 particles that were accumulated at the nonpermissive temperature. These reversibly fixed *ts*112 particles constituted a homogeneous population of 600S particles banding at 1.315 g/cm^3 in a CsCl gradient. Analysis on an SDS-polyacrylamide gel after cleavage of the cross-links revealed that they lacked core protein precursor P VII and its cleavage product, VII. The other core protein, V, was also poorly

represented. In contrast, they contained two major polypeptide species, 50K and 39K. A minor band of 32,000 daltons was detected in the particles at a late stage after shift-down (Fig. 12). They also contained a small fragment of DNA, with a sedimentation coefficient ranging from 7 to 11S. Electron microscopy confirmed the homogeneity of the fixed *ts*112 particle population: it showed round particles containing an inner structure different from the core of a mature virion. It is impossible to assess, from the available data, whether the 39K protein corresponds to the nuclear phosphorylated 39K described in Ad5 (21) or to the early 39,000- to 40,000-dalton protein reported at early stages of infection by Ad2 (7, 22). However, preliminary results indicate that both 50K and 39K are phosphorylated within the *ts*112 1.315-g/cm^3 particles (not shown).

These *ts*112 particles banding at 1.315 g/cm^3 thus differed significantly in morphology and polypeptide composition from the empty particles obtained in CsCl gradients with WT (30) and with *ts*112 in the absence of fixation (Fig. 2b). These particles resembled the structures described as the prohead for bacteriophage P 22 (2, 15). They were tentatively termed "light IM." This value of 1.315 g/cm^3 (and, therefore, the ratio of DNA to protein content) was higher than that previously reported for empty and incomplete particles isolated without prior fixation (8, 20). This buoyant density was close to that of a class of top components with a buoyant density of 1.306 g/cm^3 (14) and to that of a class of IM particles found at 1.30 g/cm^3 in CsCl after glutaraldehyde fixation of nuclear intermediates extracted from nuclei by sonic treatment (11). Unfortunately, the irreversible nature of glutaraldehyde fixation does not permit any further analysis of the protein and DNA content of these 1.30-g/cm^3 particles and renders impossible comparison with the *ts*112 IM.

Because there is no ideal method available for nuclear particle extraction, there might be objections that either the 1.315- or the 1.30-g/cm^3 or both classes of particles represent fragmentation products of true intermediates existing in vivo. However, the same light 1.315-g/cm^3 particles are found in Ad2 WT as precursors of heavy IM, containing a normal viral DNA and preceding the young virions (9). In addition, the results of pulse-chase experiments in the presence of cycloheximide indicated that at least a fraction of these *ts*112 1.315-g/cm^3 particles were able to evolve into mature virions upon the shift-down to permissive temperature. All these data suggest that these light particles represent a real intermediate step in the adenovirus assembly pathway.

It has been shown that Ad2 WT IM (which probably consist of several classes of particles of different densities) contain major bands of precursor P VII, core protein V, and protein 50K. This latter protein is released upon maturation of intermediates into young virions (11). The fact that *ts*112 IM particles contain two labeled nonvirion polypeptides, 50K and 39K, further supports the hypothesis that the *ts*112 IM particles may be precursors to the Ad2 WT IM previously described (11). This hypothesis is compatible with our results showing that, upon temperature shift-down, the release of both 39K and 50K occurs simultaneously with the entry of proteins V and P VII and DNA to form young virions. This is also compatible with the results of pulse-chase labeling kinetics of Ad2 WT particles. This shows that light, 1.315-g/cm^3 particles precede "heavy IM" of 1.37 g/cm^3 of buoyant density, which, themselves, precede young virions, banding at 1.345 g/cm^3 (9).

Either 39K or 50K or both might, thus, serve as scaffolding protein(s), and the following tentative sequence of events for the adenovirus maturation process is proposed: virus capsid components $\rightarrow$ light IM (ρ = 1.315 g/cm^3) $\rightarrow$ heavy IM (ρ = 1.37 g/cm^3) $\rightarrow$ young virion (ρ = 1.345 g/cm^3) $\rightarrow$ mature virion (ρ = 1.345 g/cm^3). The *ts*112 lesion is apparently located on the exit of either 39K or 50K or both, and/or on the encapsidation of viral DNA. The exact determination of the nature and position of this *ts* lesion on the adenovirus genome requires further genetic and biochemical studies.

The occurrence of minute amounts of *ts*112 IM, similar to the heavy IM observed in WT (9), between light IM and young virions upon temperature shift-down suggests that packaging of viral DNA with the entry of V and P VII on the one hand and the exit of 39K and 50K on the other hand is a rapid process, as already observed (28). It is, thus, impossible to determine whether the entry of viral DNA precedes or follows the entry of V and P VII or if these two processes are simultaneous. Experimental data obtained with Ad2 WT (9) suggests that DNA entry occurs after the release of 39K and 50K and before the entry of core protein V and precursor P VII. This hypothesis is also supported by the recent finding of the absence of P VII-VII in the core structures preexisting outside of the virus capsid (13).

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ADDENDUM

After completion of this study, a paper was published describing the characterization of an Ad2 *ts* mutant blocked on DNA encapsidation at an early stage of empty particles at 1.30 g/cm^3, possibly preceding the 1.315-g/cm^3 IM (G. Khittoo and J. Weber, Virology **81**:126-137, 1977).

LITERATURE CITED

1. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. J. Virol. **12**:241-252.
2. **Botstein, D., C. H. Waddell, and J. King.** 1973. Mechanism of head assembly and DNA encapsulation in *Salmonella* phage P 22. I. Genes, proteins, structures and DNA maturation. J. Mol. Biol. **80**:669-695.
3. **Boulanger, P. A., and F. Puvion.** 1973. Large-scale preparation of adenovirus soluble antigens in a highly purified form. Eur. J. Biochem. **39**:37-42.
4. **Boulanger, P. A., and F. Puvion.** 1974. Adenovirus assembly: cross-linking of adenovirus type 2 hexons in vitro. Eur. J. Biochem. **43**:465-470.
5. **Burlingham, B. T., D. T. Brown, and W. Doerfler.** 1974. Incomplete particle of adenovirus. I. Characteristics of the DNA associated with incomplete adenovirions of types 2 and 12. Virology **60**:419-430.
6. **Casjens, S., and J. King.** 1975. Virus assembly. Annu. Rev. Biochem. **44**:555-611.
7. **Chin, W. W., and J. V. Maizel, Jr.** 1977. The polypeptides of adenovirus. VIII. The enrichment of E 3 (11,000) in the nuclear matrix fraction. Virology **76**:79-89.
8. **Daniell, E.** 1976. Genome structure of incomplete particles of adenovirus. J. Virol. **19**:685-708.
9. **D'Halluin, J.-C., G. R. Martin, G. Torpier, and P. A. Boulanger.** 1978. Adenovirus type 2 assembly analyzed by reversible cross-linking of labile intermediates. J. Virol. **26**:357-363.
10. **Doerfler, W.** 1969. Nonproductive infection of baby hamster kidney cells (BHK 21) with adenovirus type 12. Virology **38**:587-606.
11. **Edvardsson, B., E. Everitt, H. Jörnvall, L. Prage, and L. Philipson.** 1976. Intermediates in adenovirus assembly. J. Virol. **19**:533-547.
12. **Eiserling, F. A., and R. C. Dickson.** 1972. Assembly of viruses. Annu. Rev. Biochem. **41**:467-502.
13. **Everitt, E., S. A. Meador, and A. S. Levine.** 1977. Synthesis and processing of the precursor to the major core protein of adenovirus type 2. J. Virol. **21**:199-214.
14. **Ishibashi, M., and J. V. Maizel, Jr.** 1974. The polypeptides of adenovirus. V. Young virions, structural intermediates between top components and aged virions. Virology **57**:409-424.
15. **King, J., E. V. Lenk, and D. Botstein.** 1973. Mechanisms of head assembly and DNA encapsulation in *Salmonella* phage P 22. II. Morphogenetic pathway. J. Mol. Biol. **80**:697-731.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (London) **227**:680-685.
17. **Öberg, B., J. Saborio, T. Persson, E. Everitt, and L. Philipson.** 1975. Identification of the in vitro translation products of adenovirus mRNA by immunoprecipitation. J. Virol. **15**:199-207.
18. **Philipson, L., K. Lonberg-Holm, and U. Pettersson.** 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2**:1064-1075.
19. **Prage, L., S. Höglund, and L. Philipson.** 1972. Structural proteins of adenoviruses. VIII. Characterization of incomplete particles of adenovirus type 3. Virology **49**:745-757.
20. **Rosenwirth, B., S. Tjia, M. Westphal, and W. Doerfler.** 1974. Incomplete particles of adenovirus. II. Kinetics of formation and polypeptide composition of adenovirus type 2. Virology **60**:431-437.
21. **Russel, W. C., and G. E. Blair.** 1977. Polypeptide phosphorylation in adenovirus-infected cells. J. Gen. Virol. **34**:19-35.
22. **Saborio, J. L., and B. Öberg.** 1976. In vivo and in vitro synthesis of adenovirus type 2 early proteins. J. Virol. **17**:865-875.
23. **Sundquist, B., E. Everitt, L. Philipson, and S. Hoglund.** 1973. Assembly of adenoviruses. J. Virol. **11**:449-459.
24. **Tjia, S., E. Fanning, J. Schick, and W. Doerfler.** 1977. Incomplete particles of adenovirus type 2. III. Viral and cellular DNA sequences in incomplete particles. Virology **76**:365-379.
25. **Traut, R. R., A. Bollen, T. T. Sun, J. W. B. Hershey, J. Sundberg, and L. R. Pierce.** 1973. Methyl 4-mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the Escherichia coli 30S ribosomes. Biochemistry **12**:3266-3273.
26. **Wallace, R. D., and J. Kates.** 1972. State of adenovirus 2 deoxyribonucleic acid in the nucleus and its mode of transcription: studies with isolated viral deoxyribonucleic acid-protein complexes and isolated nuclei. J. Virol. **9**:627-635.
27. **Warocquier, R., and P. A. Boulanger.** 1976. Adenovirus protein maturation at 42°C. J. Gen. Virol. **30**:233-242.
28. **Weber, J.** 1976. Genetic analysis of adenovirus type 2. III. Temperature sensitivity of processing of viral proteins. J. Virol. **17**:462-471.
29. **Williams, J. F., M. Gharpure, S. Ustacelebi, and S. McDonald.** 1971. Isolation of temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. **11**:95-101.
30. **Winberg, G., and G. Wadell.** 1977. Structural polypeptides of adenovirus type 16 incomplete particles. J. Virol. **22**:389-401.
31. **Winters, W. D., and W. C. Russel.** 1971. Studies on assembly of adenovirus in vitro. J. Gen. Virol. **10**:181-194.
32. **Wrigley, N. G., and H. G. Pereira.** 1974. In vitro reconstitution, hexon bonding and handedness of incomplete adenovirus capsid. J. Mol. Biol. **85**:617-631.