Characterization of a Temperature-Sensitive Fiber Mutant of Type 5 Adenovirus and Effect of the Mutation on Virion Assembly

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A temperature-sensitive, fiber-minus mutant of type 5 adenovirus, H5ts142, was biochemically and genetically characterized. Genetic studies revealed that H5ts142 was a member of one of the three apparent fiber complementation groups which were detected owing to intracistronic complementation. Recombination analyses showed that it occupied a unique locus at the right end of the adenovirus genetic map. At the nonpermissive temperature, the mutant made stable polypeptides, but they were not glycosylated like wild-type fiber polypeptides. Sedimentation studies of extracts of H5ts142-infected cells cultured and labeled at 39.5°C indicated that a limited number of the fiber polypeptides made at the nonpermissive temperature could assemble into a form having a sedimentation value of 6S (i.e., similar to the trimeric wild-type fiber), but that this 6S structure was not immunologically reactive. When H5ts142-infected cells were shifted to the permissive temperature, 32°C, fiber polypeptides synthesized at 39.5°C were as capable of being assembled into virions as fibers synthesized in wild type-infected cells; de novo protein synthesis was not required to allow this virion assembly. In H5ts142-infected cells incubated at 39.5°C, viral proteins accumulated and aggregated into particles having physical characteristics of empty capsids. These particles did not contain DNA or its associated core proteins. However, when the infected culture was shifted to 32°C, DNA appeared to enter the empty particles and complete virions developed. The intermediate particles obtained had the morphology of adenoviruses, but they contained less than unit-length viral genomes as measured by their buoyant density in a CsCl density gradient and the size of their DNA as determined in both neutral and alkaline sucrose gradients. The reduced size of the intermediate particle DNA was demonstrated to be the result of incompletely packaged DNA molecules being fragmented during the preparative procedures. Hybridization of labeled DNA extracted from the intermediate particles to filters containing restriction fragments of the adenovirus genome indicated that the molecular left end of the viral genome preferentially entered these particles.

In recognition of the information gained from studies with conditional lethal mutants of bacteriophages (17, 21, 29, 33, 34), temperature-sensitive (*ts*) mutants of adenoviruses, predominantly types 2, 5, and 12, have been isolated and characterized to complement and extend the biochemical analyses of the replication and assembly of adenovirus (26). A number of conditionally lethal *ts* mutants of adenoviruses have been isolated in this laboratory (20) to explore the interaction between virus and cell under both permissive and nonpermissive conditions and to study various aspects of viral replication and assembly.

Very little is known about the mechanism of assembly of adenoviruses. In productive infec-tion several classes of particles are produced. The various particles differ from mature virions in polypeptide composition and buoyant density in CsCl gradients. One class consists of capsids without DNA and is referred to as empty particles or top components (30, 40, 45, 47). These particles lack polypeptides V and VII, which correspond to the two core proteins, and contain some polypeptides not present in virions (30, 45, 52). Another class, which bands between the empty particles and mature virions in CsCl gradients, contains viral DNA sequences shorter than full-length virion DNA (3, 8–10, 30, 45, 47, 54, 58) and has been referred to as incomplete particles (45). All of these particles have been suggested to represent intermediates in the as-
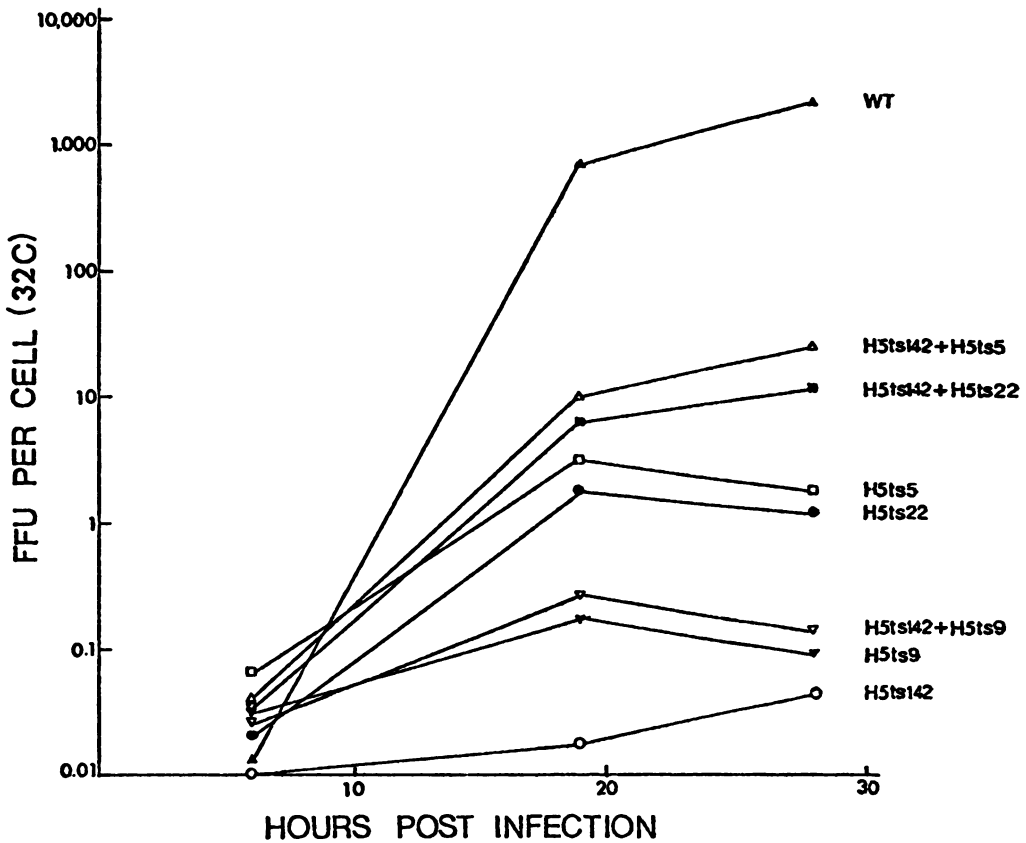
FIG. 1. Complementation between H5*ts*142 and three fiber-minus *ts* mutants of Ad5. Suspension cultures of KB cells were infected with 15 PFU of each mutant per cell in pairs, with one mutant alone or with WT virus, and incubated at 39.5°C. Samples were withdrawn from cultures at indicated times, frozen and thawed to release intracellular virus, and titrated at 32°C, using the fluorescent focus assay. FFU, Focus-forming units.

sembly of adenovirus based on pulse-chase labeling kinetics of the different particles in vivo (18, 30, 52) and on in vitro association studies (55).

ts mutants of type 2 and 5 adenoviruses (Ad2 and Ad5) have been used in the study of viral assembly (4, 10, 11, 19, 59). Some of the mutants used do not accumulate intermediates (4, 11), others form labile intermediates that have to be cross-linked or fixed and analyzed under mild conditions (10, 18, 19), and all of the DNA-containing intermediates obtained have shorter than unit-length DNA (10).

This paper reports the biochemical and genetic characterization of H5*ts*142, an Ad5 *ts* mutant defective in the synthesis of fiber antigen, and the use of this mutant to elucidate the pathway of assembly of adenovirus virions. It is demonstrated that empty capsids accumulate in mutant-infected cells at the nonpermissive temperature, that upon shift-down particles into which viral DNA appears to be entering are detected,

that the molecular left end of the viral DNA appears to enter the empty capsid first during assembly, and that these intermediate particles are probably precursors of infectious virions.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of KB cells were grown in modified Eagle minimal essential medium (15, 16, 41) supplemented with 10% human or horse serum. The cells were suspended in the same medium (minimal essential) containing 5% calf serum before viral infection. Monolayer cultures of KB cells were grown in regular minimal essential medium supplemented with 10% calf serum and were used in some experiments under maintenance medium (infecting fluid; 25). HeLa cells (62) were propagated in monolayer cultures in Eagle basal medium supplemented with 10% calf serum.

Viral stocks of the Ad5 wild-type (WT) strain and mutants were prepared in suspension cultures as previously described (20). Plaque-purified stocks of *ts* mutants were tested for viral titer and reversion frequency before use. Virus was assayed by means of
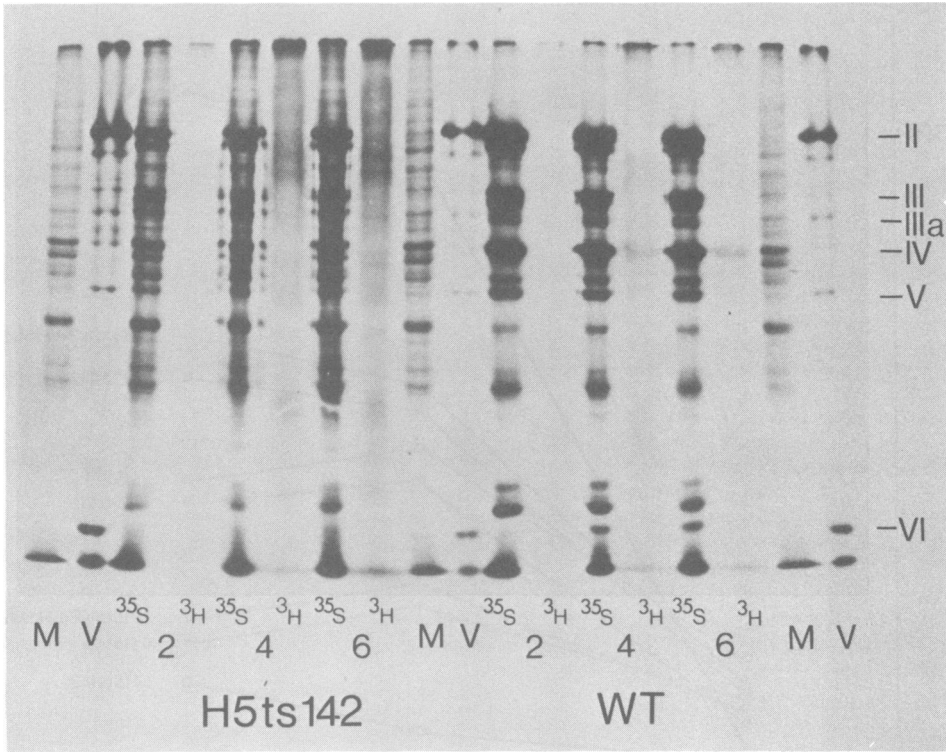
FIG. 2. SDS-polyacrylamide gel autoradiogram of [³H]glucosamine [³H]- or [³⁵S]methionine (³⁵S)-labeled cell extracts from WT- and H5ts142-infected cells incubated at 39.5°C. KB cells in monolayers were infected with 100 PFU of WT or H5ts142 trypsin-treated virus per cell at 39.5°C and incubated for 18 h. The cells were then labeled with 100 μCi of [³H]glucosamine or 50 μCi of [³⁵S]methionine per ml for 2 to 6 h at 39.5°C. Monolayers were washed with phosphate-buffered saline at the end of the labeling period and harvested in phosphate-buffered saline. The washed cells were disrupted in sample buffer and boiled for 2 min. Electrophoresis was carried out at 70 V on a 10% SDS–polyacrylamide slab gel. V, Purified virion; M, mock-infected cells labeled for 15 min with [³⁵S]methionine. The numbers under the sample slots represent the labeling times.

plaque assay (36, 62) and indirect immunofluorescence assay (fluorescent focus assay), as described previously (20, 44, 53).

Genetic and protein structure studies. Complementation and genetic recombination experiments were performed as previously described (20) except that HeLa cell monolayers were used for plaque assays (62). Complementation was considered positive if the mixed infection was about 10-fold above the sum of the yields of the singly infected cultures. Recombination frequencies are expressed as: $[(2\times \text{ yield titrated at } 38.8°C/\text{yield titrated at } 32°C)] \times 100$. Multiplication by the factor 2 takes into account the undetected double ts mutations which are assumed to arise at a frequency equal to that of the ts⁺ class. The left- and right-hand ends of the genetic map are oriented according to the physical map of adenovirus as determined by Doerfler and Kleinschmidt (12).

Conditions for radioactive labeling and viral assembly studies. To label KB cells in monolayers with [³H]glucosamine and [³⁵S]methionine for glycosylation studies, 3×10^6 cells per plate were washed with Hanks balanced salt solution and labeled for 2 to 6 h at 39.5°C with [³H]glucosamine (100 μCi/ml, final concentra-

tion) containing 1% serum–1% glucose in regular minimal essential medium or with [³⁵S]methionine (50 μCi/ml, final concentration) in low-methionine medium (31). At the end of the labeling period, the cells were washed with ice-cold phosphate-buffered saline, harvested, and stored as a cell pellet at −20°C.

To pulse-label cells in temperature shift experiments, KB cells in suspension cultures were centrifuged at 1,000 × g for 15 min. The cells were then resuspended to a density of 2×10^6 per ml in spinner-minimal essential medium containing 1/20 the normal concentration of nonradioactive methionine and 5% calf serum and incubated for 1 h at 39.5°C to deplete the pools. [³⁵S]methionine (25 to 40 μCi/ml, final concentration), [³H]thymidine (30 to 50 μCi/ml, final concentration), or a combination of both was then added, and incubation was continued for 30 min to 2 h at 39.5°C before shifting to 32°C.

For analyzing radioactively labeled infected cell extracts in viral assembly studies, extracts were layered on preformed linear CsCl density gradients and centrifuged at 22,500 rpm and 4°C for 3 h in an SW41 rotor. After centrifugation, 15-drop fractions were collected from the bottom of the tube. A sample of
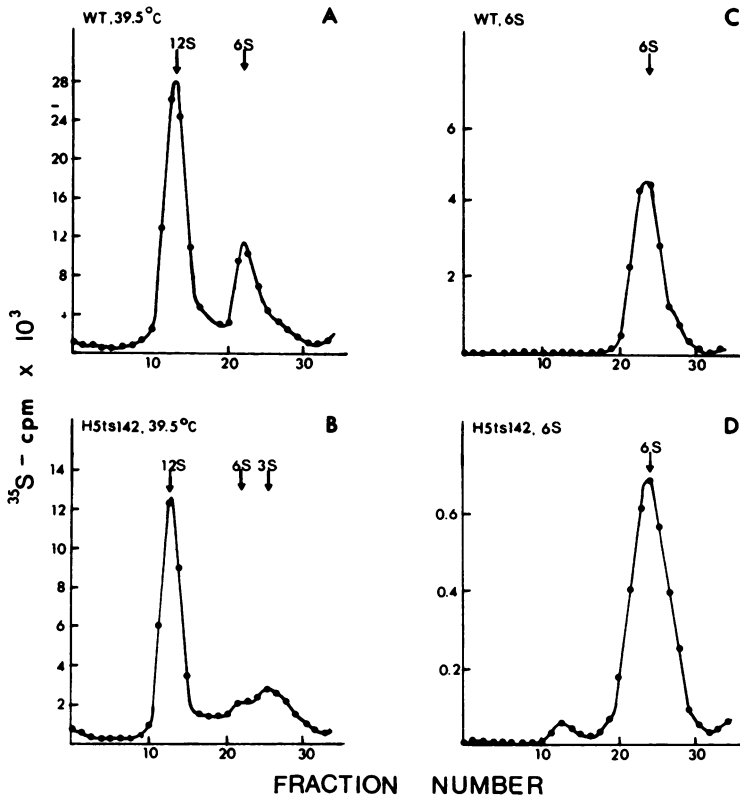
FIG. 3. Synthesis of viral capsomers in WT- and H5*ts*142-infected cells at 39.5°C. Two suspension cultures of KB cells were infected with 100 PFU of either virus per cell and incubated at 39.5°C for 18 h after which 50 μCi of [^{35}S]methionine per ml was added for 30 min. Cells were sedimented by centrifugation, washed in cold phosphate-buffered saline, and resuspended to a concentration of 10^7 cells per ml in gradient buffer (0.15 M NaCl, 0.001 M EDTA, 0.01 M sodium phosphate buffer, pH 7.6) containing 100 μg each of RNase and DNase per ml. The cell suspensions were sonicated for 10 s and then incubated for 1 h at 37°C. A 0.2-ml portion of each extract was placed onto a 10 to 30% (wt/vol) linear sucrose gradient and centrifuged for 23 h at 35,000 rpm in a Beckman SW41 rotor at 4°C. A ^{3}H-labeled fiber marker was used. The 6S fractions of the gradients (A and B) were subjected to another velocity sedimentation in 10 to 30% sucrose gradient for further purification (C and D). Sedimentation is from right to left.

each fraction was spotted onto a 3MM filter pad (Whatman) and processed. The filters were dried and radioactivity was measured in a liquid scintillation spectrometer.

Studies of synthesis of viral capsomers. A modification of the method described by Velicer and Ginsberg (57) was used to determine assembly of adenovirus capsomers in infected cells. Extracts of [^{35}S]methionine-labeled infected KB cells were prepared and layered on 10 to 30% linear sucrose gradients. The gradients were centrifuged at 4°C for 23 h at 35,000 rpm in an SW41 rotor (Beckman). Hexon and fiber antigens were assayed by immunoprecipitation as previously described (56, 57).

Preparation of antisera. The immunization of rabbits with purified hexon and fiber has been described elsewhere (14, 56, 57).

Polyacrylamide gel electrophoresis. In preparation, cell pellets were resuspended directly in gel sample buffer (1% sodium dodecyl sulfate [SDS], 1% 2-β-mercaptoethanol, 50 mM Tris-chloride, pH 6.8, and 10% glycerol), or viruses were dialyzed against sample buffer overnight at room temperature; samples were boiled for 2 min before electrophoresis. Ten and 7.5% discontinuous polyacrylamide gels were prepared by using the buffer system of Laemmli (35). Slab gels were prepared in an apparatus described by Studier (51) with a 4% stacking gel. Ten micrograms or less of protein per well was electrophoresed at 70 to 100 V until the dye front reached the bottom of the gel. Gels were stained with Coomassie brilliant blue (Fisher Scientific Co.) and then dried under vacuum, followed by autoradiography with Kodak Royal X-mat X-ray film. Densitometer scanning of radioautograms at 540 nm was done with a Gilford spectrophotometer equipped with a linear transport attachment.

Viral multiplication. Single-step viral growth curves were determined in suspension cultures as previously described (32). Samples (2 ml) were removed at various times, subjected to six cycles of freezing and thawing to release intracellular virus, and titrated for infectivity by means of a fluorescent focus assay.

TABLE 1. Immunoprecipitation of peak fractions of the 12S (hexon) and 6S (fiber) regions from sucrose gradients[a]

Samples		Total cpm	Specific cpm precipitated[b]	% Specific precipitation
Peak	Virus			
12S	WT	274,854	267,708	97.4
	H5ts142	110,622	100,666	91
6S	WT	34,735	31,262	90
	H5ts142	5,559	334	6

[a] Immune precipitation was performed on samples of the peak fractions from the 12S and 6S regions of sucrose gradients (Fig. 3), using specific antihexon and antifiber antisera, respectively, followed by goat anti-rabbit gamma globulin.

[b] Specific precipitation was calculated from the total precipitation by subtraction of nonspecific trapping in samples in which normal rabbit serum was substituted for either antihexon or antifiber antisera.

Viral purification. Adenovirus was purified from infected KB cells similar to the method described previously (36). Infected cells were incubated in the presence of 0.5% sodium deoxycholate and 20 μg of DNase per ml for 30 min at 37°C. The mixture was then centrifuged, and the supernatant was extracted three times with equal volumes of Freon 113 (trichlorotrifluoroethane; Dupont). The aqueous phase was centrifuged through a discontinuous CsCl gradient consisting of 1.2 g of CsCl per ml on top of 1.4 g of CsCl per ml for 3 h at 22,500 rpm. The viral band was collected and centrifuged through a preformed linear gradient of CsCl (density, 1.2 to 1.4 g/ml) in an SW41 rotor for 16 h at 22,500 rpm at 4°C. The viral band was again collected and stored at 4°C.

Density gradients. Linear 5 to 20% sucrose density gradients were used to determine the sedimentation characteristics of the labeled viral DNA at neutral and alkaline pH values. Purified virions labeled with [³H]thymidine were lysed by the addition of 0.5 ml of 5% deoxycholate on top of 5 to 20% sucrose gradients containing 0.01 M Tris–1 M NaCl–0.19 N NaOH–0.001 M EDTA, pH 7.6. The gradients were centrifuged in an SW27.1 rotor at 22,000 rpm for 18 h. Fractions were collected and the radioactivity was determined as described above. Viral DNA (extracted as described below) was analyzed by centrifugation (20,000 rpm for 18 h at 4°C in an SW27.1 rotor) through a 5 to 20% neutral sucrose gradient containing 0.01 M Tris–0.1 M NaCl–0.001 M EDTA, pH 7.6. Fractions were collected and the radioactivity was determined. [¹⁴C]thymidine-labeled adenovirus DNA extracted from purified virions of Ad5 was included in all gradients as an internal marker.

Extraction of viral DNA. Purified virions or incomplete particles, obtained by CsCl density gradient centrifugation (36), were dialyzed against a buffer containing 0.05 M Tris-hydrochloride (pH 7.2)–0.005 M EDTA. Dialyzed virions and particles derived from 2 × 10⁷ cells were adjusted to a final volume of 1 ml by the addition of the above buffer. SDS (final concentration, 0.5%) and pronase (final concentration, 0.5 mg/ml; Calbiochem) were added, and the mixture was

incubated for 1 h at 37°C. Viral DNA was extracted from disrupted virions by extracting three times with redistilled phenol saturated with the above buffer (2). Generally, 50 to 100 μg of viral DNA was recovered from particles purified from 2 × 10⁷ infected KB cells.

Restriction endonuclease treatments. Ad5 DNA prepared from virions was digested with restriction endonucleases HindIII and EcoRI as previously described (13).

Agarose gel electrophoresis of DNA. DNA fragments were separated by preparative electrophoresis on 1% agarose gels (3 mm thick, 340 mm long, and 160 mm wide) by a procedure similar to that of Peacock and Dingman (42). Samples containing 75 to 100 μg of DNA in 100 μl were electrophoresed at 10 to 12 mA until the dye marker was about 1 cm from the bottom edge of the gel. After electrophoresis, the gel was removed from the apparatus and immersed for 5 min in 0.1% ethidium bromide made up in buffer, and the DNA fragments were visualized with a hand-held UV light source.

Preparation of Ad5 fragments. Slices of agarose gels were cut so that each contained an individual fragment, and the DNA fragment was recovered by treatment with sodium perchlorate and chromatography on a hydroxyapatite column (38). Samples dialyzed against 0.01 M Tris-hydrochloride (pH 7.2)–0.001 M EDTA were serially extracted once with redistilled phenol and three times with ether and then precipitated in cold ethanol. One microgram of the recovered fragment was rerun on an analytic 1% agarose gel to determine purity. Samples were found to be >90% pure.

Preparation of filters for DNA hybridization. Filters containing DNA fragments were prepared as previously described (39).

DNA-DNA hybridization. Hybridization of ³H-labeled DNA to filter-bound viral DNA was performed as described in earlier studies (5). A total of 5,000 cpm of ¹⁴C-labeled, purified, adenovirus-denatured DNA was also added to each sample to serve as an internal control.

Isotopes and chemicals. [³⁵S]methionine (300 to 500 Ci/mmol), [³H]glucosamine (5 to 15 Ci/mmol), and [³H]thymidine were purchased from New England Nuclear Corp. Fluorescein-conjugated goat anti-rabbit antiserum was obtained from Hyland Laboratories. Cycloheximide was purchased from Sigma Chemical Co., and restriction endonucleases HindIII and EcoRI were from Biolabs and Miles Laboratories, respectively.

RESULTS

Genetic analyses. Complementation studies performed on all ts mutants isolated in our laboratory indicated that H5ts142 was the single member of a nonoverlapping complementation group defective in the synthesis of immunologically reactive fiber at the nonpermissive temperature, 39.5°C (20). Williams et al. (63) also isolated ts mutants of Ad5, and they reported that fiber-minus mutants were members of three complementation groups. Complementation studies were done with ts142 and representative ts mutants from each of the three complementa-
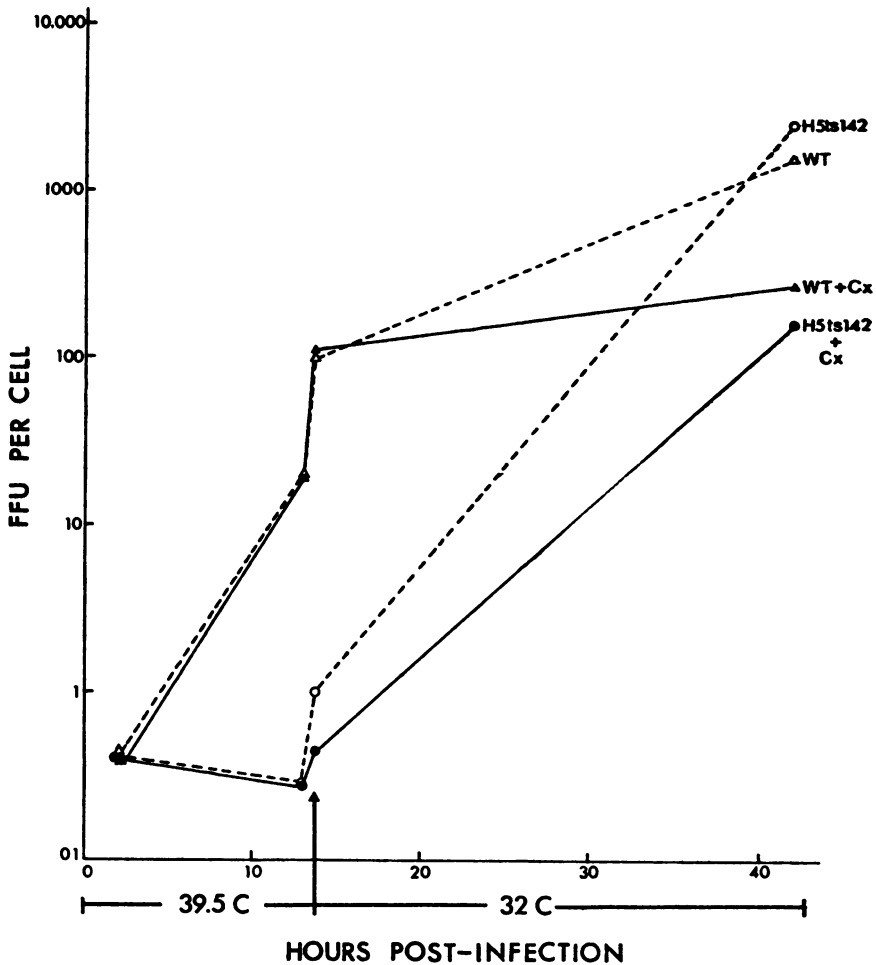
FIG. 4. Viral multiplication after a shift of H5*ts*142-infected cells from 39.5 to 32°C. Suspension cultures of KB cells were infected with 20 PFU of either WT or H5*ts*142 virus per cell and incubated at 39.5°C. At 13 h postinfection (indicated by arrow), the cells were labeled with [^{35}S]methionine (50 μCi/ml, final concentration) for 15 min at 39.5°C. After a 30-min chase period at 39.5°C, each culture was divided equally into two fresh spinner flasks and incubated at 32°C. A WT- and a H5*ts*142-infected culture were each treated with 10 μg of cycloheximide (Cx) per ml at the time of temperature shift. Samples were withdrawn at the times indicated, frozen and thawed to release intracellular virus, and titrated for infectivity at 32°C, using the fluorescent focus assay. FFU, Focus-forming units.

tion groups of mutants isolated in Glasgow: H5*ts*5, H5*ts*9, and H5*ts*22 (kindly supplied by J. F. Williams). The results of these experiments (Fig. 1) indicated that H5*ts*142 and H5*ts*9 failed to complement each other and were assigned to the same complementation group. Marker rescue analysis also indicated that the mutations of H5*ts*142 and H5*ts*9 were close to each other (unpublished data). Double infections of H5*ts*142 with H5*ts*5 and H5*ts*22, the other fiber mutants, produced infectious virus, but the yield was only about 10 times the higher of the two yields of the single infection at 39.5°C. It should be noted that single infections with H5*ts*5 and

H5*ts*22 also produced viral yields 30- to 100-fold greater than the eclipse titer. These complementation analyses suggested that all of the fiber mutants belonged to a single complementation group and that intracistronic complementation (7, 22) occurred in mixed infections of *ts*142 × *ts*5 or *ts*22.

Recombination studies with pairwise crosses of the fiber mutants indicated that they mapped very closely together. The recombination frequencies between H5*ts*142 and H5*ts*9, between H5*ts*142 and H5*ts*22, and between H5*ts*142 and H5*ts*5 were 0.017, 0.122, and 0.19, respectively, and that between H5*ts*22 and H5*ts*5 was 0.283.

TABLE 2. Assembly into virions of polypeptides labeled with [^{35}S]methionine at 39.5°C after a shift to 32°C[a]

Virus	Ratio (by wt)					
	II/II	III/II	IIIa/II	IV/II	V/II	VI/II
WT	1	0.2813	0.2220	0.2276	0.3357	0.3235
H5ts142	1	0.2431	0.1545	0.2464	0.3690	0.3131

[a] The *ts* radioactive labeled cultures described in the legend to Fig. 4 were incubated at 32°C for 30 h after the shift from 39.5°C to allow maximum virion production. The cells were harvested by centrifugation, and virions were purified from cultures incubated without cycloheximide. The purified virions were disrupted in gel sample buffer containing 1% SDS–1% β-mercaptoethanol–0.05 M Tris-hydrochloride buffer (pH 6.8)–10% glycerol and boiled for 2 min. Electrophoresis was carried out at 70 V on a 10% polyacrylamide–SDS slab gel. The radioautograms were scanned, and the proportions of radioactively labeled fibers recovered from the virions in WT- and H5ts142-infected cells were compared by weighing the appropriate areas under the curves formed from the densitometry tracing of the autoradiograms. The values were normalized by comparing with the value for proteins II (hexon), III (penton base), IV (fiber), V (core protein), and VI (hexon-associated protein).

Two-factor crosses with mutants located outside the fiber region placed the fiber mutants very near to the right-hand end of the genome.

Modification of fiber polypeptides. Pulse-chase experiments with [^{35}S]methionine to label H5ts142- and WT-infected cell polypeptides at the nonpermissive temperature indicated that fiber polypeptides were made and remained stable during a 6-h chase in H5ts141-infected cells (data not shown). It had been reported by Ishibashi and Maizel (31) that the fiber polypeptide, whether integrated into the virion or not, has a sugar residue related to glucosamine linked to the polypeptide chain with a weak alkaline-sensitive bond. It was therefore determined whether H5ts142-infected cells incubated at 39.5°C synthesized glycosylated fiber polypeptides. At 18 h postinfection, monolayers of KB cells were labeled with [^{3}H]glucosamine or [^{35}S]methionine for 2 to 6 h at 39.5°C. At the end of the labeling period, samples were prepared for SDS-polyacrylamide gel electrophoresis. Figure 2 shows the radioautogram of a 10% gel containing electrophoresed mutant- and WT-infected cell extracts labeled with [^{3}H]glucosamine or [^{35}S]methionine. In the WT-infected culture labeled with [^{3}H]glucosamine, polypeptide IV (fiber) was labeled in 4 h, and the intensity of the label increased with increased labeling time. In H5ts142-infected cells, the fiber polypeptides were made but their structure appeared affected so that incorporation of [^{3}H]glu-

cosamine could not be detected even in a 6-h period (even prolonged exposure of the radioautogram did not detect labeling). It should also be noted that host protein synthesis was not shut off in H5ts142-infected cells at 39.5°C, unlike that observed with WT virus.

Synthesis of viral capsomers. In the initial immunofluorescence studies (20), H5ts142 was found to be defective in the synthesis of immunologically reactive fibers at the nonpermissive temperature. To determine whether the 6S trimers, which form the fibers, were assembled (14), velocity sedimentation analysis of radioactively labeled, infected-cell extracts was carried out in sucrose gradients (57). The findings for the WT-infected culture (Fig. 3A) are similar to those reported earlier (57). In the H5ts142-infected culture incubated at 39.5°C (Fig. 3B), 12S particles were synthesized to about one-half the amount made in WT-infected cells, but the 6S particles were produced in even smaller amounts. The radioactive material having a sedimentation coefficient of approximately 3.4S represented the unassembled polypeptide chains of viral capsomers (57).

To estimate the structural fidelity of material that sedimented in the 6S region of sucrose gradients of WT- and H5ts142-infected cells labeled at 39.5°C, the peak fractions of the 6S regions of sucrose gradients of WT- and H5ts142-infected cell extracts were subjected to another velocity sedimentation (Fig. 3C and D) before immune coprecipitation and SDS-polyacrylamide gel electrophoretic analysis. Immune coprecipitation was carried out with rabbit anti-fiber antiserum. Peak fractions of the 12S regions of sucrose gradients of WT- and H5ts142-infected cell extracts were also subjected to immune coprecipitation with rabbit antihexon serum for comparison (Table 1). Normal rabbit serum was used in place of the specific antihexon and antifiber antisera in duplicate samples to quantify the amount of nonspecific trapping. Hexons from Wt- and H5ts142-infected cells labeled at the nonpermissive temperature were both almost quantitatively precipitated (Table 1). However, there was a marked decrease in immunoprecipitation of the protein from the 6S region of the gradient of H5ts142-infected cells, which would contain any assembled fibers labeled at the nonpermissive temperature. These data indicate that in H5ts142-infected cells incubated at the nonpermissive temperature the protein sedimenting as 6S material either did not have the native immunological reactivity of fiber or was not fiber.

One-step growth curve after temperature shift. To investigate whether the *ts* gene product synthesized at 39.5°C could renature and function at 32°C, the effect of a temperature shift on the
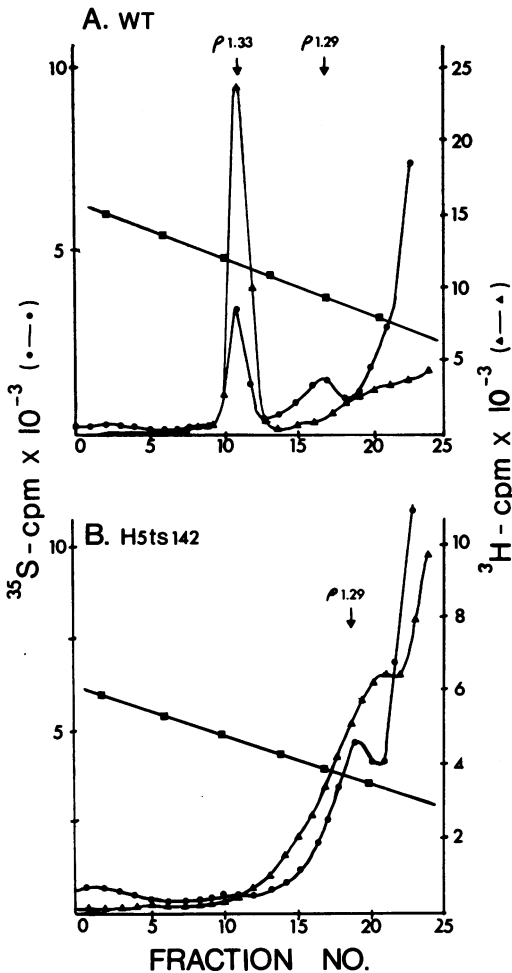
FIG. 5. Equilibrium CsCl density gradient analysis of particles in WT- and H5*ts*142-infected cells incubated at 39.5°C. KB cells in suspension cultures were infected with 100 PFU of WT or H5*ts*142 per cell at 39.5°C and labeled from 6 to 24 h postinfection with 2.5 µCi each of [^{35}S]methionine and [^{3}H]thymidine per ml. Viral suspensions were obtained from labeled infected cells and Freon extracted, as described in the text. The Freon extracts were layered on preformed linear CsCl gradients ρ 1.2 to 1.4 g/ml and centrifuged in an SW41 rotor for 16 h at 22,500 rpm and 4°C. Fractions were collected and the radioactivity was determined. Symbols: ●, [^{35}S]methionine; ▲, [^{3}H]thymidine; ■, density (grams per milliliter).

production of infectious viral particles was studied. The need for protein synthesis for viral production after the temperature shift was also examined by studying the effect of inhibition of protein synthesis with cycloheximide (10 µg/ml) at the time of temperature change. At 13 h postinfection at 39.5°C, the cells were labeled for 15 min with [^{35}S]methionine, and the radioactively labeled infected cells were cultured in unlabeled medium for 30 min at 39.5°C before the cultures were shifted to 32°C. This chase period depleted the cellular pool of radioactive [^{35}S]methionine available for protein synthesis and allowed any unassembled polypeptide chains to be completed and possibly converted to capsomers before the temperature was lowered. The labeled cultures were then equally divided into two fresh spinner flasks and incubated at 32°C. One WT- and one H5*ts*142-infected culture were each treated with 10 µg of cycloheximide per ml at the time of lowering the temperature. The cultures were incubated at 32°C for 30 h to allow the maximum production of virions. The virus was then purified from cultures incubated without cycloheximide. The purified virions were disrupted and electrophoresed in polyacrylamide gels containing SDS. The radioautograms were scanned, and the proportion of radioactively labeled fibers recovered from the virions in WT- and H5*ts*142-infected cells was accomplished by weighing the appropriate areas under the curves formed from the densitometer tracings of the radioautograms of disrupted virions.

The results showed that, on shift down to 32°C (Fig. 4), the final viral yield attained in H5*ts*142-infected cells was equal to that of cells infected with WT virus. Moreover, the final yields of both virus-infected cultures were reduced about 10-fold when incubated in the presence of cycloheximide. Therefore, the formation of infectious virions in H5*ts*142-infected cultures after a shift to 32°C did not require continuous protein synthesis. The analysis of the radioautogram densitometry tracings (Table 2) indicated that a similar proportion of radioactivity, compared with hexons, was present in fibers and the other capsid polypeptides recovered from WT and H5*ts*142 virions after the long chase period. Thus, polypeptides that accumulated at 39.5°C in H5*ts*142-infected cells could form mature fibers, and they could also be efficiently used in virion assembly at 32°C.

Characterization of particles in infected cells incubated at 39.5°C. In H5*ts*142-infected cells incubated at 39.5°C, some fiber polypeptides synthesized were assembled into 6S forms, but they were not immunologically reactive (Fig. 3; Table 1). Experiments were designed to determine whether the multimeric fiber polypeptide structures could interact with other capsid proteins and whether some type of particles could result from these interactions. At 6 h after infection at 39.5°C, cells were labeled with [^{35}S]methionine and [^{3}H]thymidine for 17.5 h. A cell extract was prepared and analyzed on preformed linear CsCl gradients of density (ρ) 1.2 to 1.4 g/ml.

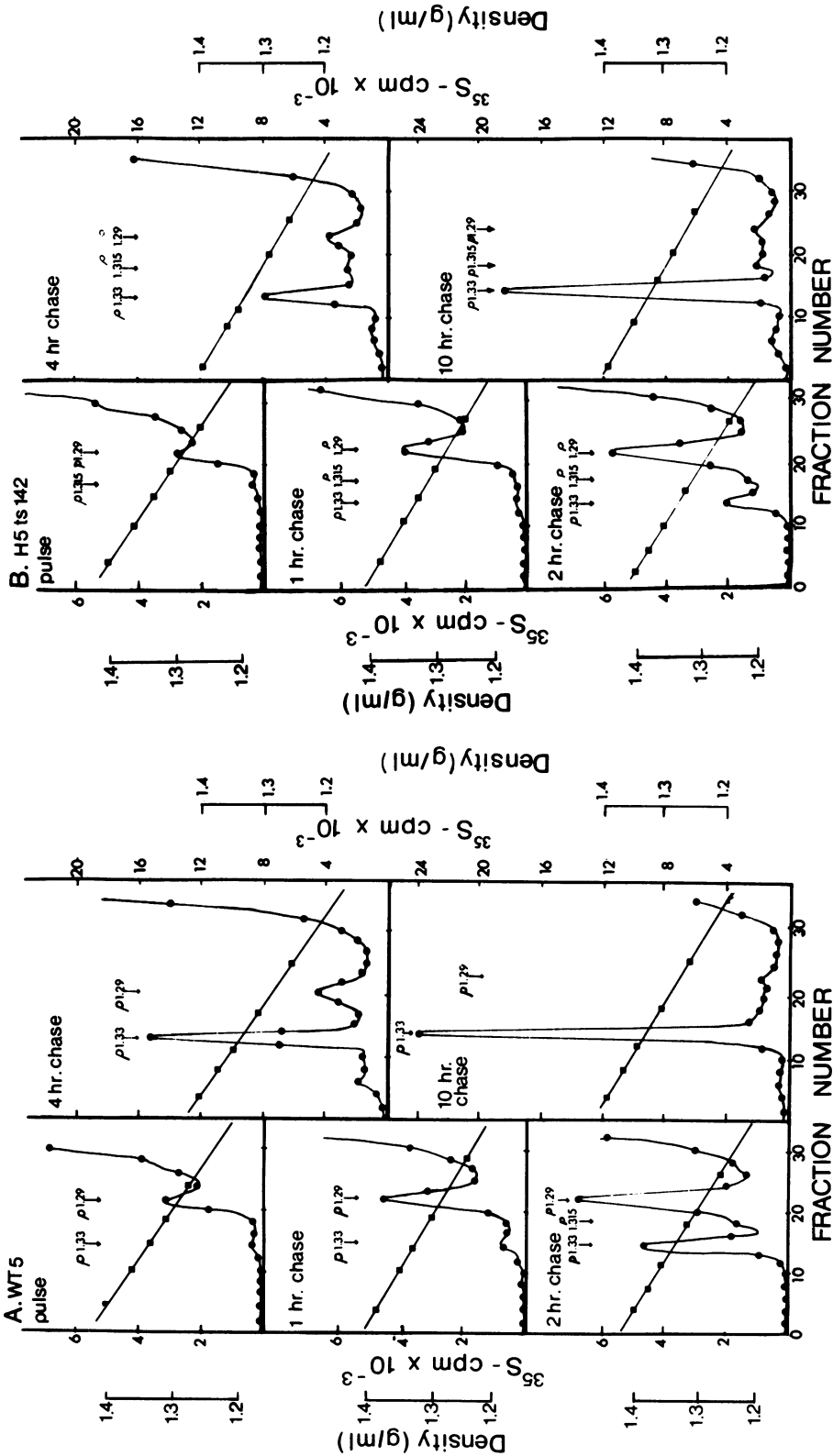In WT-infected cells incubated at 39.5°C (Fig.

FIG. 6. Analysis of adenovirus particles in (A) WT- and (B) H5ts142-infected cells after shift from 39.5 to 32°C. KB cells in suspension cultures were infected with 20 PFU of WT or H5ts142 per cell and incubated at 39.5°C for 12.5 h. The cells were then resuspended in medium containing 5% of the normal concentration of methionine, incubated for another hour at 39.5°C, and then labeled at 39.5°C for 30 min with [35S]methionine (30 μCi/ml, final concentration). The labeled cells were sedimented by centrifugation and resuspended in medium containing a 10-fold increase of unlabeled methionine. The cultures were shifted to 32°C and incubated for various periods of time. Samples were taken at indicated times for 10 h during the chase and analyzed on preformed linear CsCl gradients (ρ 1.2 to 1.4 g/ml) as described in the text. Symbols: ●, [35S]methionine; ■, density.

5A), labeled materials banded with buoyant densities of 1.33 and 1.29 g/ml. These bands corresponded to the buoyant densities of complete virions and the so-called top component, respectively. Only the complete virions contained DNA as reflected by labeling with [³H]thymidine. In H5*ts*142-infected cells incubated at 39.5°C (Fig. 5B), only material banding at a buoyant density of 1.29 g/ml was detected. No comparable radioactive thymidine peak was observed, implying that these "top components" did not contain DNA. Electrophoresis in SDS-polyacrylamide gels of the material that banded at a buoyant density of 1.29 g/ml (electropherogram not presented) revealed that they did not contain polypeptides V and VII, the DNA-associated virion core proteins, but there were present polypeptides having the characteristics of pVI, pVII, and pVIII, the precursors to polypeptides VI, VII, and VIII, respectively. The polypeptide composition, density, and lack of nucleic acid of these particles suggested that they were empty particles (30, 40, 45, 47). Electron microscopic examination and sedimentation in sucrose gradients (unpublished data) further indicated that the proteins having a buoyant density of 1.29 g/ml in WT- and H5*ts*142-infected cells were empty particles.

Pulse-chase and shift-down studies of WT- and H5*ts*142-infected cell cultures. The possibility that the empty capsids in H5*ts*142-infected cells incubated at 39.5°C were viable intermediates in the assembly pathway of Ad5 was investigated. At 13.5 h after infection at 39.5°C, cells were labeled for 30 min with [³⁵S]methionine, after which the cultures were shifted to 32°C. At various times after the shift, samples were taken and extracts were prepared for CsCl density gradient analysis. Immediately after the pulse, in WT-infected cells (Fig. 6A), radioactivity was detected in particles having the electron microscopic appearance and the buoyant density of empty capsids (ρ 1.29 g/ml) and complete virions (ρ 1.33 g/ml) (30, 40). The empty capsids, however, were present in greater abundance. The radioactivity in empty capsids increased rapidly up to 2 h after the pulse with [³⁵S]methionine and then decreased. The radioactivity in complete virions increased at a comparatively slower rate for the first 2 h after the pulse, and it then rapidly increased from 2 to 10 h after the onset of the chase. In H5*ts*142-infected cells (Fig. 6B), radioactivity was detected in the particles with buoyant densities of 1.29 and 1.315 g/ml immediately after the pulse. The radioactivity of the empty particles (buoyant density, 1.29 g/ml) increased rapidly up to 2 h after the initiation of the chase period and then decreased. Particles with a buoyant density of complete virions (ρ 1.33 g/ml) were detected in very small amounts

after only 1 h of chase; they steadily increased in quantity from 1 to 4 h and then were the predominant forms after 10 h of chase. The radioactivity in particles with a buoyant density of 1.315 g/ml increased slowly from 1 to 4 h of chase and then reached a constant level. These findings suggested that the empty and intermediate particles were formed before virions and might be the precursors of mature virions.

Characterization of intermediate particles. To characterize the particles having an intermediate density of 1.315 g/ml according to their DNA content, experiments similar to those described in Fig. 6 were carried out, except that cells were labeled with [³H]thymidine as well as with [³⁵S]methionine. In both WT- and H5*ts*142-infected cultures (Fig. 7), [³H]thymidine was incorporated into structures banding at buoyant densities of 1.315 and 1.33 g/ml, which were possibly intermediate and complete particles, respectively. In H5*ts*142-infected cells (Fig. 7B), radioactivity increased in the ρ 1.315-g/ml particles for up to 2 h and then remained relatively constant. Assembly of complete virions (ρ 1.33 g/ml) appeared to occur slower during the first 60 min of the chase period than thereafter. In WT-infected cells, since assembled complete virions increased considerably after the first hour of chase, intermediate particles were not detected thereafter.

The materials that banded at densities of 1.315 and 1.33 g/ml in H5*ts*142-infected cells, which corresponded to the position of intermediate and complete particles, respectively, were examined by electron microscopy. The particles in each band had the characteristic icosahedral structure (not shown). Most of the particles of ρ 1.33 g/ml appeared to be complete virions which excluded the stain; a few were partially penetrated by stain, and some appeared to have been disrupted, probably during preparation. In contrast, the phosphotungstic acid appeared to have penetrated the capsids of the majority of particles having intermediate buoyant densities. The data indicate that particles that banded with a buoyant density of 1.33 g/ml had the characteristic structure of infectious adenovirus virions and that particles with a buoyant density of 1.315 g/ml were incomplete particles with less than the usual amount of DNA (so-called intermediate particles).

Analysis of these particles on SDS-polyacrylamide gel electrophoresis (Fig. 8) indicated that polypeptides V and VII could not be detected in the intermediate particles until 4 h of chase. Polypeptide VI and the precursor polypeptides to protein VI and VII (pVI and pVII, respectively) were present. Protein VIII's precursor (pVIII) was not clearly separated from pVI. These particles were similar in polypeptide com-
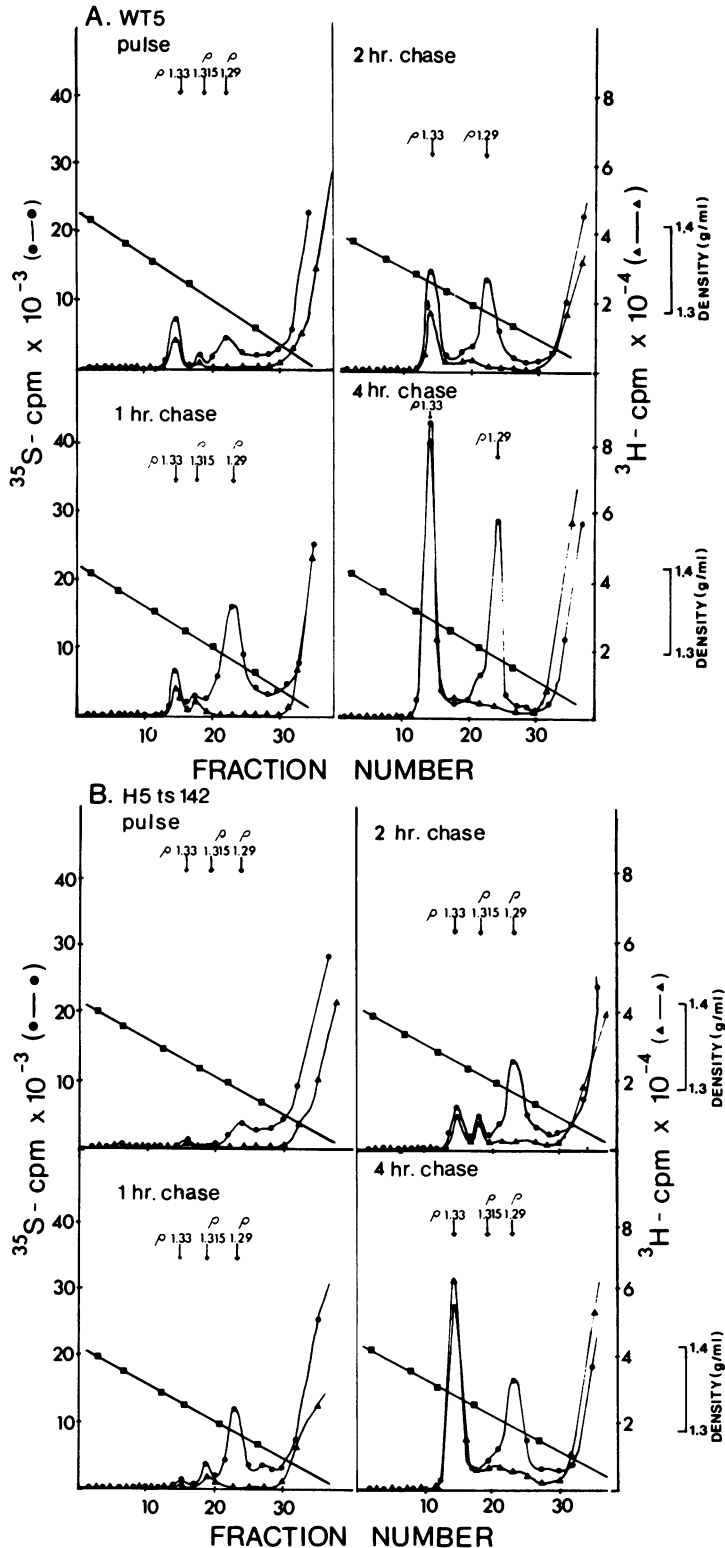
FIG. 7. Analyses, after temperature shift to 32°C, of assembly into particles containing polypeptides and DNA synthesized at 39.5°C in cells infected with (A) WT and (B) H5ts142. KB cells in suspension cultures were infected and incubated exactly as described in the legend to Fig. 6 except that cells were labeled for 30 min with 30 μCi each of both [^{35}S]methionine and [^{3}H]thymidine per ml. The cells were sedimented and resuspended in medium containing a 10-fold increase of unlabeled methionine. The cultures were then shifted to 32°C, and incubation was continued for various periods of time. Samples were taken at the indicated times during the chase period, disrupted by sonication, and analyzed on preformed linear CsCl gradients (ρ 1.2 to 1.4 g/ml) as described in the text. Fractions were collected and radioactivity was determined. Symbols: ●, [^{35}S]methionine; ▲, [^{3}H]thymidine; ■, density.
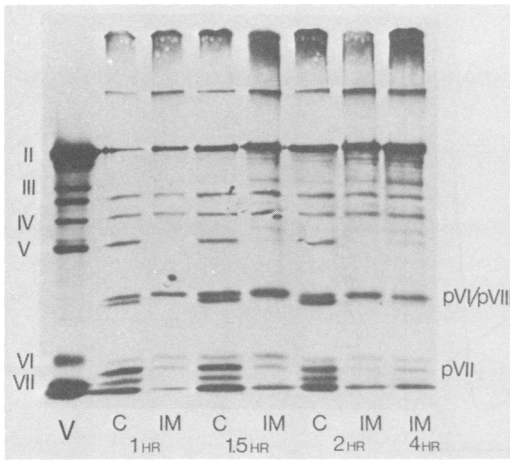
FIG. 8. SDS-polyacrylamide gel electrophoretic analyses of the particles assembled in H5*ts*142-infected cells after a temperature shift to 32°C. Materials from the complete and intermediate particles at the different times indicated during the chase were pooled separately, dialyzed against TE buffer (10 mM Tris-chloride, pH 7.2, 1 mM EDTA), disrupted in gel sample buffer, and analyzed on a 10% SDS-polyacrylamide slab gel. Autoradiogram of the gel are shown. C, Complete particles; IM, intermediate particles; V, virion.

position to the intermediate particles described by Edvardsson et al. (18).

Characteristics of DNA associated with intermediate particles. When H5*ts*142-infected cell cultures were shifted from 39.5°C to the permissive temperature, [³H]thymidine radioactivity appeared to enter first into icosahedral particles having a buoyant density of 1.315 g/ml (Fig. 7). Intermediate particles which contained viral sequences shorter than unit-length DNA have been described previously (3, 45, 58). Experiments were carried out to determine whether the incomplete DNA in the intermediate particles was an artifact of preparation and whether it represented a unique portion of the viral genome, since it was unclear why assembly intermediates could contain DNA of less than unit length. These experiments were similar to those described above except that the cells were only labeled with [³H]thymidine for 30 min at 39.5°C and chased at 32°C for 2 h. The labeled viral materials obtained by sonication of infected cells were analyzed in CsCl gradients. The complete and intermediate particles were isolated in CsCl gradients, and the size of the DNA was determined on alkaline (Fig. 9) and neutral sucrose gradients (data not presented). The results from both types of gradients indicated that DNA isolated from the particles of intermediate density was smaller in size than unit-length DNA.

Since the isolation procedure for these parti-

cles involved sonication to release viral material from infected cells, the reduction in size of the DNA in the ρ 1.315-g/ml particles might have resulted from the shearing of incompletely packaged DNA during preparation procedures. Therefore, the more gentle method of freezing and thawing was chosen to release viral material from infected cells to investigate this possibility.

Figure 10A shows the CsCl density gradient pattern for a lysate of H5*ts*142-infected cells prepared by freeze-thawing cells after 2 h of chase at the permissive temperature. Labeled materials banded broadly, with apparent peaks at buoyant densities of 1.33 and 1.34 g/ml. The freeze-thawed labeled material to be analyzed was extremely viscous, which might account for the aberrant broad peaks observed in these gradients. The material banding at a buoyant density of 1.34 g/ml was taken to represent complete virions. The material banding at a buoyant density of 1.33 g/ml was assumed to be particles with incompletely packaged DNAs, resulting in lower buoyant densities. The particles with buoyant densities of 1.34 and 1.33 g/ml were pooled separately and centrifuged to equilibrium in CsCl gradients. The size of the DNAs in these particles were determined on alkaline sucrose gradients (Fig. 10B and C). The results indicated that DNAs in particles in both peaks were of unit length. These data suggested that the reduction in size of DNA isolated from particles of ρ 1.315 g/ml, released by sonic treatment of cells, was due to fragmentation of incompletely packaged DNA associated with viral particles during assembly. Additional evidence was provided when a portion of the material containing each type of purified particle was sonicated and then analyzed on equilibrium CsCl gradients. The data show that after sonication the buoyant density of purified particles was converted from 1.34 to 1.33 g/ml (Fig. 11B and D), suggesting that the higher buoyant density was due to material (probably DNA) associated with the virion surface. Moreover, particles with a buoyant density of 1.33 g/ml disappeared after sonication, and instead particles with a buoyant density of 1.315 g/ml were present (Fig. 11C and E). These findings support the interpretation that the so-called intermediate particles were indeed particles into which only a portion of the viral DNA had entered and that the buoyant density of 1.315 g/ml was the result of shearing during preparation of the samples.

DNase treatment of complete and intermediate particles obtained after sonication of labeled H5*ts*142-infected cell lysate showed that only the DNA associated with intermediate particles was sensitive to DNase treatment. These data (not presented) further imply that the DNA associated with intermediate particles was in-
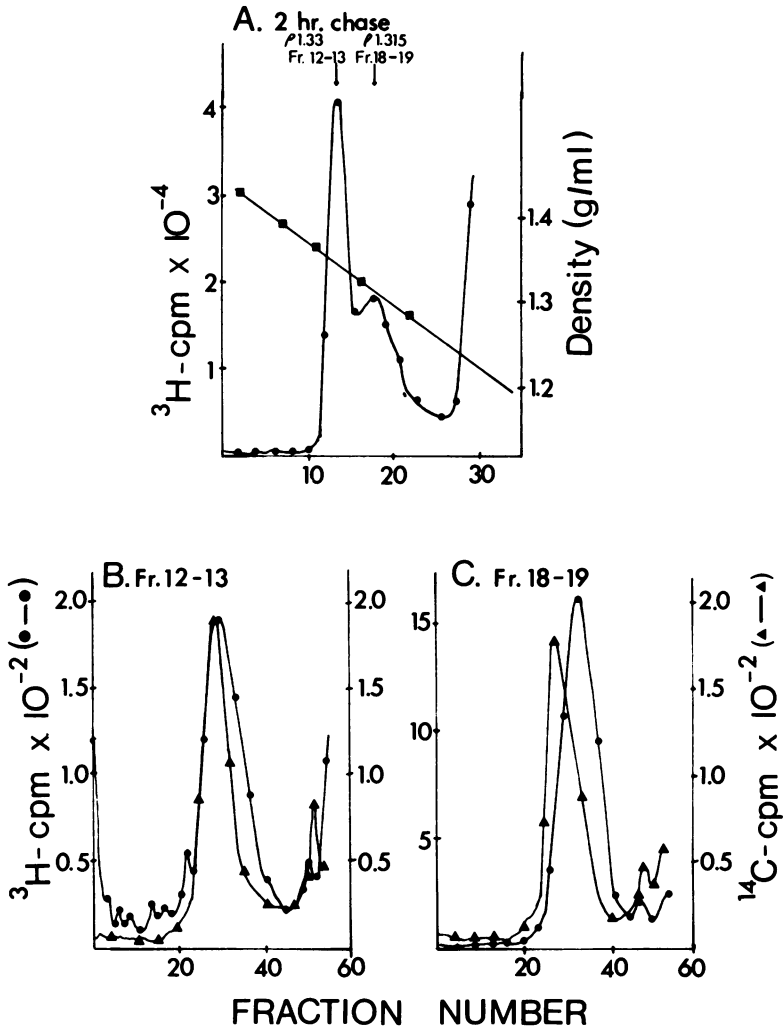
FIG. 9. Alkaline sucrose gradient centrifugation of [^{3}H]DNA isolated from complete and intermediate particles prepared from sonicated, infected cells. H5ts142-infected cells labeled with [^{3}H]thymidine for 30 min at 39.5°C and chased at 32°C for 2 h were harvested by centrifugation, resuspended in TE buffer, sonicated two times, and centrifuged to equilibrium in preformed linear CsCl gradients (ρ 1.2 to 1.4 g/ml) as described in the text. The "heavy" (fractions [Fr] 12 and 13) and intermediate (fractions 18 and 19) fractions from the CsCl gradient (A) were pooled separately, dialyzed against TE buffer, and layered on 5 to 20% alkaline sucrose gradients containing 1 M NaCl–0.19 N NaOH–0.001 M EDTA–0.01 M Tris (pH 7.6), and 0.5 ml of 0.5% deoxycholate was then added. ^{14}C-labeled DNA isolated from purified Ad5 was used as a marker. The gradients were centrifuged for 18 h at 20,000 rpm and 4°C in an SW27.1 rotor. Fractions were collected and the radioactivity was determined as described in the text. Sedimentation was from right to left. Symbols: ●, [^{3}H]thymidine; ▲, [^{14}C]thymidine; ■, density.

completely packaged unit-length DNA which was sensitive to both DNase and sonic treatments.

Region of viral genome present in incomplete viral particles. To determine whether the DNA of less than viral genome length represented a unique region of the viral DNA that initially associated with and entered the preformed capsids, [^{3}H]thymidine-labeled DNAs from both

complete and intermediate particles obtained after sonication of infected cells were analyzed by hybridization to filters containing specific fragments of the adenovirus genome. If a specific region of the DNA were involved in the initial interaction with these particles, that DNA should be protected from shearing during preparative procedures and could be identified by hybridization to filters containing specific DNA
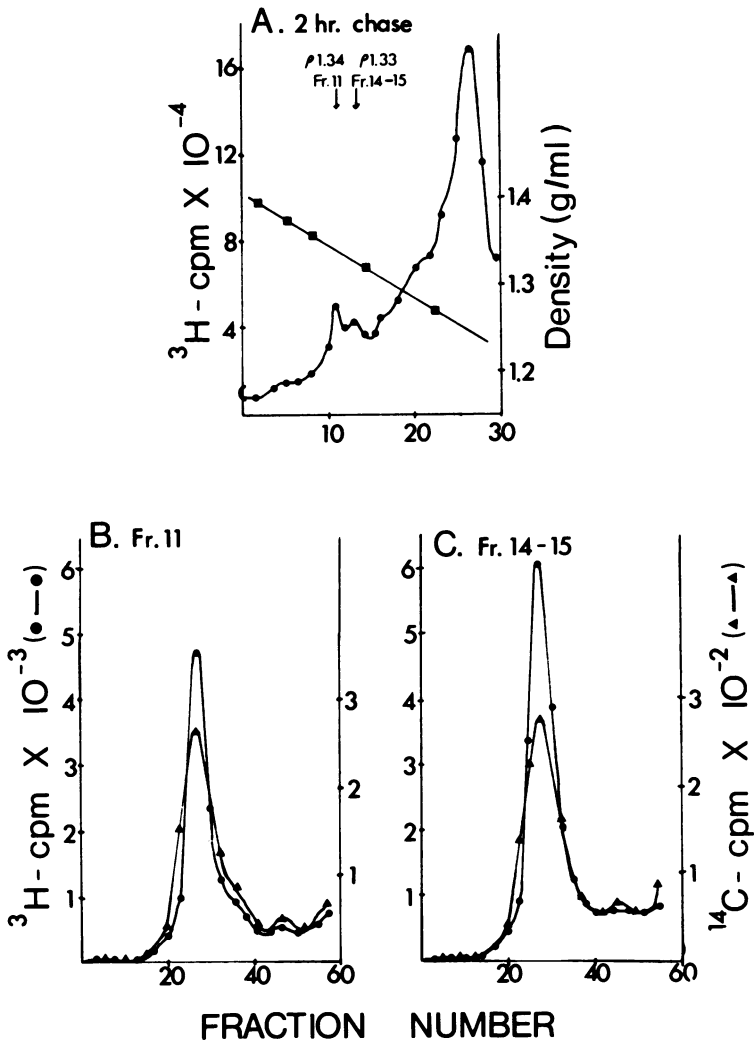
FIG. 10. Alkaline sucrose gradient centrifugation of [^{3}H]DNA isolated from heavy particles released from infected cells by freezing and thawing. H5*ts*142-infected cells labeled with [^{3}H]thymidine were prepared as described (Fig. 9). The labeled infected cells were harvested, freeze-thawed six times to release viral particles, and analyzed in preformed linear CsCl gradients (ρ 1.2 to 1.4 g/ml). The two peaks (1.34 and 1.33 g/ml) from CsCl gradient were pooled separately and certrifuged to equilibrium in CsCl gradients. Purified 1.34- and 1.33-g/ml particles were dialyzed against TE buffer and layered on 5 to 20% alkaline sucrose gradients (see Fig. 9). ^{14}C-labeled DNA isolated from purified Ad5 was used as a marker. Fractions were collected and the radioactivity was determined as described in the text. Sedimentation was from right to left. Symbols: ●, [^{3}H]thymidine; ▲, [^{14}C]thymidine; ■, density.

restriction fragments. The preparation of complete and intermediate particles and the extraction and purification of DNA from these particles were performed as previously described. To prepare filters for hybridization, DNA was extracted from purified Ad5 virions and subjected to endonuclease cleavage with *Hin*dIII and *Eco*RI restriction enzymes (Table 3). The results of hybridization analyses with [^{3}H]thymidine-labeled DNA extracted from complete and

intermediate particles are summarized in Table 3. These data demonstrate that the intermediate particles, like the complete particles (virions), contained viral DNA. Moreover, these findings indicate that the intermediate (ρ 1.315 g/ml) particles contained DNA whose sequences were overrepresented by those at the left end of the genome and underrepresented by those at the right terminus. These data suggest that the left end of the genome preferably associates with
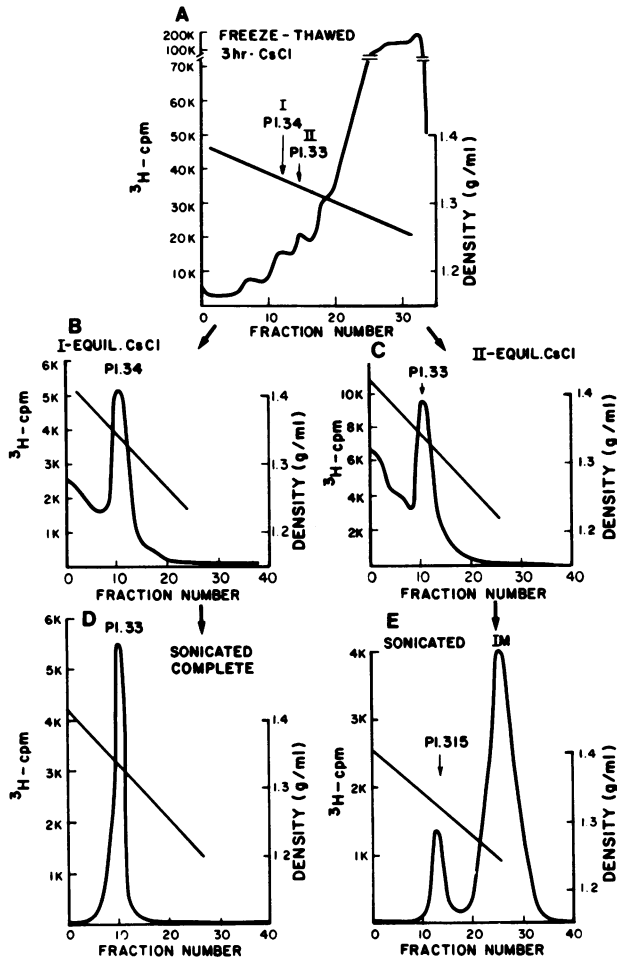
FIG. 11. Analyses of particles from freeze-thawed H5ts142-infected cells after shift from 39.5 to 32°C before and after sonication. Extract of H5ts142-infected cells labeled with [³H]thymidine was prepared as described (Fig. 10) and analyzed on a preformed linear CsCl gradient (ρ 1.2 to 1.4 g/ml). The two peaks (1.34 and 1.33 g/ml) from a CsCl gradient (A) were pooled separately and purified once more by centrifugation to equilbrium in CsCl gradients (B and C). Purified 1.34- and 1.33-g/ml particles were dialyzed against TE buffer and sonicated two times before analysis on equilibrium CsCl gradients (D and E, respectively). Fractions were collected and the radioactivity was determined.

assembling or assembled capsids and that the left end of the viral DNA first enters the empty particle.

DISCUSSION

H5ts142, a conditionally lethal ts mutant of Ad5, has been characterized biochemically and genetically. Complementation analyses with ts mutants representative of three reported complementation groups (48, 63) indicated that H5ts142 and H5ts9 were members of a nonoverlapping complementation group. The data obtained from double infections of H5ts142 with H5ts5 or H5ts22, both very "leaky" mutants, suggested that the yields of <10-fold over the

sum of the two single infections was probably the result of intracistronic complementation, which is not unusual with mutations in a gene coding for polypeptides that are assembled into multimeric proteins (7, 22). This conclusion is also consistent with recombination studies which showed that H5ts142 and H5ts9 were very close to each other and that all four fiber mutants mapped within <0.3 of a recombination unit of each other. Recombination studies with pairwise crosses of a number of ts mutants placed H5ts142 and the other fiber mutants in the right-hand portion of the adenovirus genome (26). The data are consistent with results obtained by: (i) marker rescue (23), (ii) physical mapping intertypic recombinants (6, 49), and (iii)

localization of the fiber gene by mRNA hybridization and translation studies (37, 38).

Fiber polypeptides made in H5*ts*142-infected cells apparently are not glycosylated (Fig. 2), but nevertheless they are not degraded at the nonpermissive temperature. Moreover, fiber polypeptides made at 39.5°C are assembled into a form having sedimentation value of 6S, which is similar in size to native trimeric fibers (Fig. 3), although this 6S structure is not immunologically reactive. Indeed, mutant fibers synthesized at 39.5°C are also assembled into virions to the same extent as WT fibers when the temperature is shifted to 32°C, even in the absence of protein synthesis (Fig. 4).

Mature virions are not produced in H5*ts*142-infected cells incubated at the nonpermissive temperature, but particles having the physical characteristics of empty capsids are formed. These particles, which band at a buoyant density of 1.29 g/ml, do not contain DNA or the core polypeptides V and VII. The kinetics of [^{35}S]methionine labeling in pulse-chase experiments with H5*ts*142- and WT-infected cells suggest that empty particles are formed before the assembly of complete virions. In addition, particles having a buoyant density of 1.315 g/ml appear in H5*ts*142-infected culture after a shift from 39.5 to 32°C. These particles of intermediate buoyant density have the morphology of adenoviruses and contain DNA which appears to be entering the preformed particles (Fig. 7). The so-called intermediate particles contain all of the capsid polypeptides including protein IV (albeit in reduced amount) and the precursors pVI and pVII, and probably pVIII, but the core proteins V and VII are not detectable. Proteins VI, VII, and VIII are usually processed from their precursors, pVI, pVIII, and pVII, during assembly. The polypeptide composition of these particles was similar to the intermediate particles observed in earlier studies with *ts* mutants (10, 11, 19) as well as those described as intermediates in assembly of wild-type virus (9, 18, 43). The complete particles contained polypeptides V, VI, and VII and also small amounts of the precursor polypeptides pVI, pVII, and pVIII. These were similar to the particles referred to as "young virions" (18, 30). However, since electron micrographs (unpublished data) show some intermediate particles to be present in the fractions containing complete particles, the possibility that the precursor polypeptides were contaminants could not be ruled out. The so-called young virions could be a mixture of assembled and assembling particles.

The data from pulse-chase experiments indicate that empty and intermediate particles are formed before virions and are likely to be precursors in the assembly of complete Ad5 virions.

TABLE 3. Hybridization analyses with labeled DNA from complete and intermediate particles[a]

Filters containing:	Total counts (input)				Counts hybridized				% Hybridized			
	Complete		Intermediate		Complete		Intermediate		Complete		Intermediate	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
*Eco*RI-B (83.5–100 m.u.)	11,150	1,455	11,175	1,672	2,150	255	756	270	18.6	17	6.7	16
*Hind*III-A (50.1–73.6 m.u.)	5,370	1,875	5,320	2,437	1,226	385	1,397	445	22.8	19.5	26.5	19
*Hind*III-D (41–50.1 m.u.)	5,428	2,268	5,790	2,456	687	303	1,426	314	12.5	13.4	24.6	12.8
*Hind*III-G and -E (0–17 m.u.)	11,750	1,805	8,925	2,240	2,058	197	4,618	371	17.5	11.0	52.0	16.5
Whole Ad5 DNA	10,950	1,250	3,975	1,240	11,637	851	4,686	820	100	70	100	66
λ DNA	10,060	1,275	4,575	1,275	29.5	4.6	27.6	8	0.3	0.3	0.6	0.6

[a] The individual Ad5 fragment filters were prepared as described in the text and hybridized to ^{3}H-labeled DNA isolated from complete and intermediate particles. ^{14}C counts represent marker DNA from purified virions. m.u., Map units.

An unambiguous precursor-product relationship between empty, intermediate, and complete particles cannot be established from the pulse-chase labeling kinetic studies described since the major capsid proteins accumulate in a large pool (the soluble antigen pool), and only 5 to 20% of the protein is incorporated into mature virions (27, 60, 61). However, the finding that the particles of intermediate buoyant density are probably particles into which viral DNA is only partially encapsidated presented further evidence that these particles are intermediates in the assembly pathway. Thus, the particles of buoyant density 1.315 g/ml appear to result from shearing of the viral DNA which is entering the assembling or assembled capsid. Hybridization of DNA extracted from the so-called intermediate particles to filters containing specific restriction fragments of the adenovirus genome indicate a preferred association of the molecular left-hand end of the viral genome with these particles (Table 3). Daniell (8) and Tibbetts (54) previously reported that defective interfering particles of Ad2, Ad3, and Ad7 primarily contain DNA from the left end of the viral genome. The left-hand specificity is intriguing, since the molecular ends of adenovirus DNA are identical with regard to their terminal 100- to 150-nucleotide sequences (regions of inverted terminal repetition) and the presence of the covalently bound 5'-terminus protein on each complementary strand of viral DNA (1, 24, 46, 50, 64). Recently, Hammarskjöld and Winberg (28) identified a sequence located in the first 390 to 400 base pairs of the left end of the genome of Ad16 which may contain recognition signals for encapsidation of the viral DNA. This might explain the left-hand specificity observed.

The results obtained from the assembly studies with H5ts142 suggest a model for the assembly of adenoviruses. Empty capsids, as previously proposed (30, 52), are preassembled precursors destined to interact with viral DNA molecules and their associated proteins. The molecular left hand of the viral genome, or its core proteins, contains specific recognition signals for association with empty capsids which directs the left end of the viral genome to be specifically inserted. The complete packaging of the viral genome requires substantial compression of the DNA by folding and possible displacement or cleavage processing of polypeptides associated with the viral DNA (pVII) and previously empty capsids (pVI and pVIII). Final steps in virion assembly probably require restoration or completion of external capsomers in the region where DNA enters the capsid.

These studies of a conditionally lethal ts mutant, H5ts142, not only illustrate the utility of such mutants in the study of genetic interactions such as complementation and recombination, but also indicate their value in characterizing a gene product's structural features required for its function and in revealing mechanisms regulating viral processes not solely related to the specific gene product affected.

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