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Characterization of a Temperature-Sensitive, Hexon Transport Mutant of Type 5 Adenovirus

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Infection of KB cells at 39.5°C with H5ts147, a temperature-sensitive (ts) mutant of type 5 adenovirus, resulted in the cytoplasmic accumulation of hexon antigen; all other virion proteins measured, however, were normally transported into the nucleus. Immunofluorescence techniques were used to study the intracellular location of viral proteins. Genetic studies revealed that H5ts147 was the single member of a nonoverlapping complementation group and occupied a unique locus on the adenovirus genetic map, distinct from mutants that failed to produce immunologically reactive hexons at 39.5°C ("hexon-minus" mutants). Sedimentation studies of extracts of H5ts147-infected cells cultured and labeled at 39.5°C revealed the production of 12*S* hexon capsomers (the native, trimeric structures), which were immunoprecipitable to the same extent as hexons synthesized in wild-type (WT)-infected cells. In contrast, only 3.4*S* polypeptide chains were found in extracts of cells infected with the class of mutants unable to produce immunologically reactive hexon protein at 39.5°C. Hexons synthesized in H5ts147-infected cells at 39.5°C were capable of being assembled into virions, to the same extent as hexons synthesized in WT-infected cells, when the temperature was shifted down to the permissive temperature, 32°C. Infectious virus production was initiated within 2 to 6 h after shift-down to 32°C; de novo protein synthesis was required to allow this increase in viral titer. If ts147-infected cells were shifted up to 39.5°C late in the viral multiplication cycle, viral production was arrested within 1 to 2 h. The kinetics of shutoff was similar to that of a WT-infected culture treated with cycloheximide at the time of shift-up. The P-VI nonvirion polypeptide, the precursor to virion protein VI, was unstable at 39.5°C, whereas the hexon polypeptide was not degraded during the chase. It appears that there is a structural requirement for the transport of hexons into the nucleus more stringent than the acquisition of immunological reactivity and folding into the 12*S* form.

Conditionally lethal, temperature-sensitive (ts) mutants of adenoviruses (2, 11, 19, 20, 24, 26, 35, 38, 39, 40, 49; M. J. Ensinger, Ph.D. thesis, Univ. of Pennsylvania, Philadelphia) have been isolated in recent years, with the expectation that they would prove useful in elucidating the biochemical aspects and regulatory controls of many of the reactions in viral replication and viral transformation. Among the temperature-sensitive mutants of the human type 5 and avian (CELO) adenoviruses described, several viruses (12, 19, 20, 33) show the striking characteristic that, at the nonpermissive temperature, one or more of the capsid proteins are restricted to the cytoplasm, their site of synthesis (43, 45, 46), rather than appearing in the normal nuclear position (5, 7) where viral particles are assembled late in the infectious cycle (5, 6, 30). It is not known whether these mutants are all defective in the same function, since some differences in their phenotypes have been described (19, 20, 33), but they all show the same general type of transportation defect. Recently a mutant of simian virus 40 (41, 47) has also been shown to be defective in transport of a virion antigen into the nucleus at the nonpermissive temperature. Spontaneous variants of polyoma virus (4, 18) and simian virus 40-adenovirus hybrids (PARA) (7) have been reported to have a transport deficiency.

Among the mutants of human type 5 adenovirus (12, 33), there appears to be one group with a unique defect in the transport of hexon capsomers (14). This protein, formed of three identical polypeptide chains of about 105,000 daltons (8, 27), is the major protein of the virion, making up about 90% of the viral capsid. In the infected cells, hexons are produced in vast excess of the amount found in viral particles

(28, 44, 48), and the protein accumulates in the nucleus during the viral growth cycle (5, 6, 48).

A hexon transport mutant, which was isolated in this laboratory, has been studied extensively since the existence of a class of mutants showing a transport deficiency of a single capsid protein suggests that movement of viral protein from cytoplasm to nucleus requires a distinct viral function or has stringent structural demands. Thus, elucidation of intracellular transport mechanisms, not previously amenable to study (45, 46), may now be possible. This communication presents a characterization of the conditionally lethal, temperature-

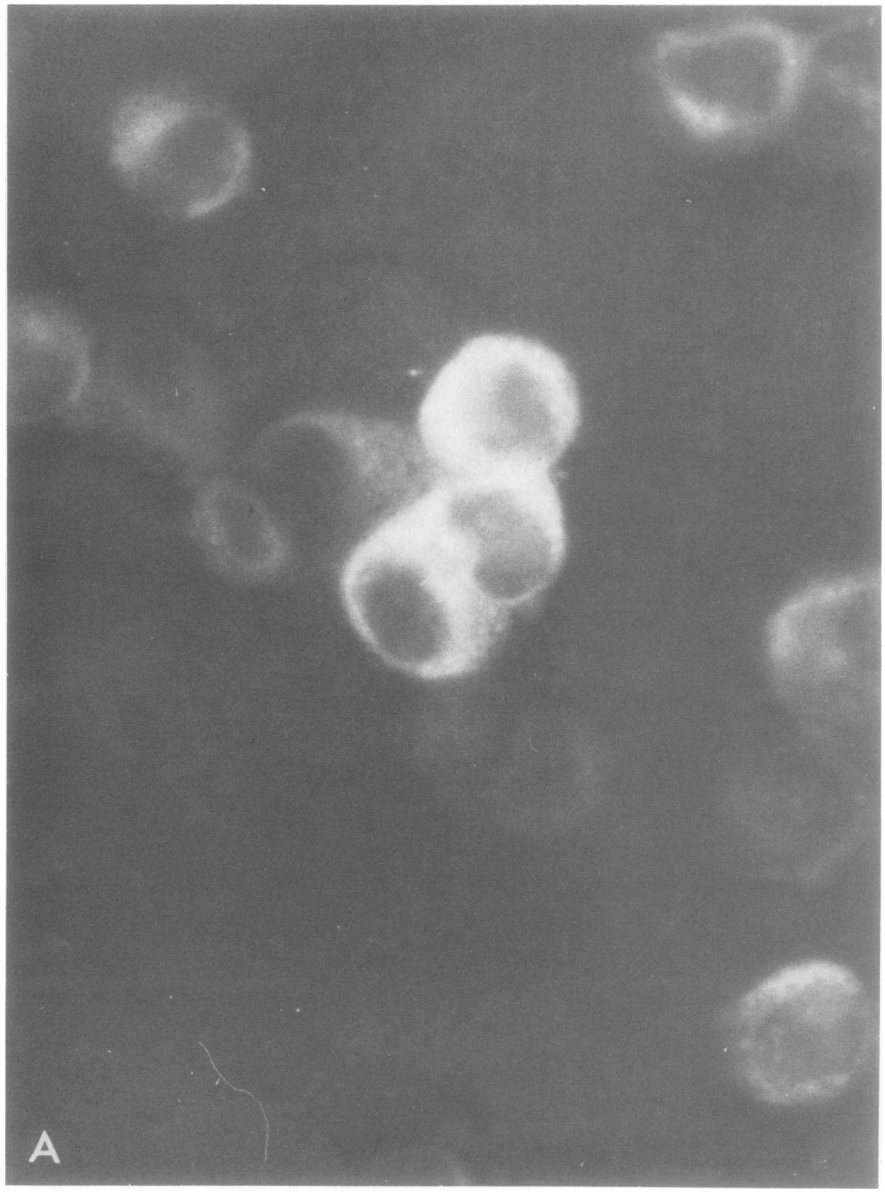
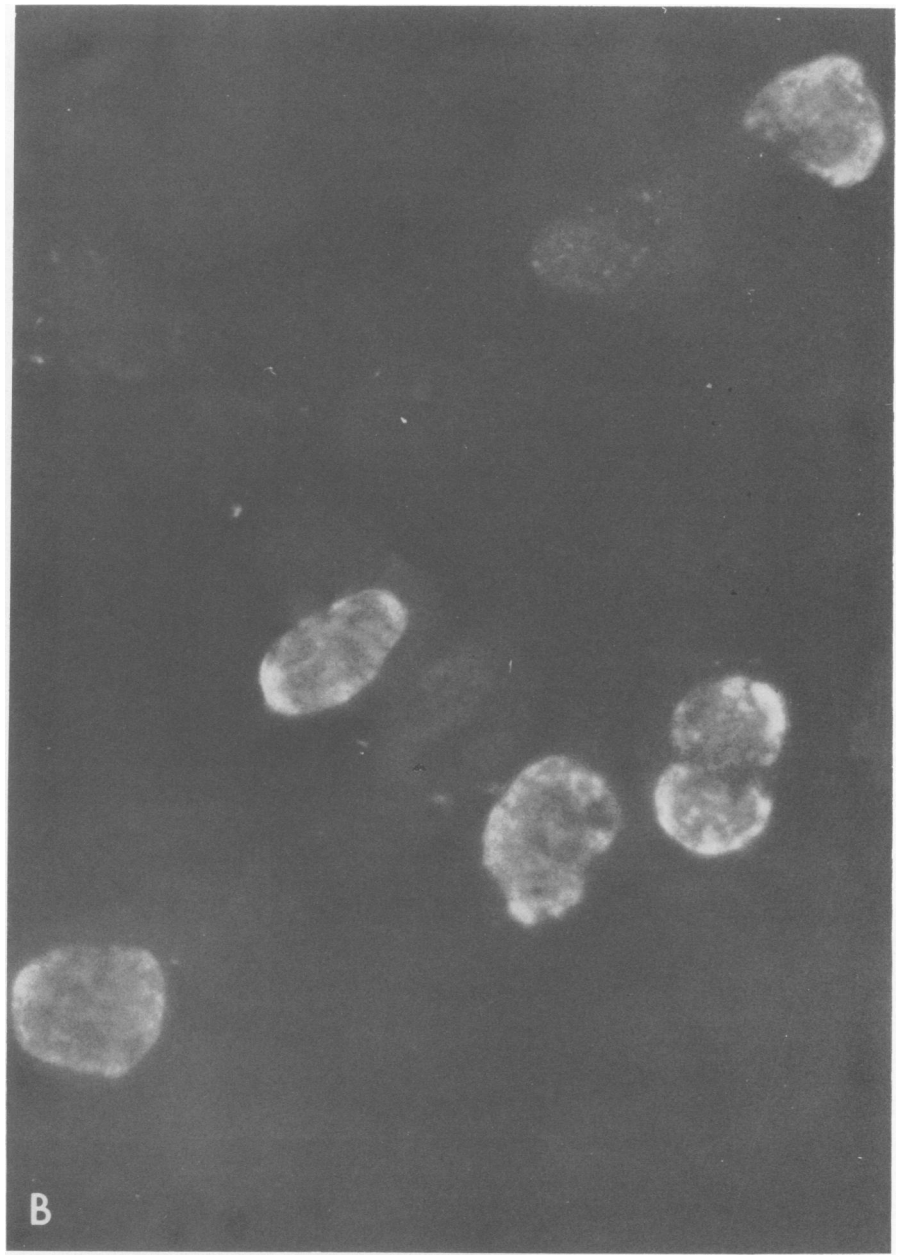


FIG. 1. *Indirect immunofluorescence of infected KB cell monolayers reacted with anti-hexon antiserum. Monolayers, grown on glass cover slips, were infected with WT and H5ts147 viruses and incubated at 39.5°C for 18 h before fixation in acetone. A two-step immunofluorescence procedure employing specific rabbit anti-hexon antiserum and fluorescein-conjugated goat anti-rabbit serum was used to localize the hexon antigen. Stained monolayers were photographed under UV illumination. ×400. (A) H5ts147-infected cells; (B) WT-infected cells.*



sensitive hexon transport mutant of type 5 adenovirus, designated H5ts147 (15).

(Preliminary reports of this investigation were presented at the 74th and 75th Annual Meetings of the American Society for Microbiology, 12–17 May 1974, Chicago, Ill., and 27 April–2 May 1975, New York, N.Y.)

MATERIALS AND METHODS

Cells and virus. KB cells were grown in suspension culture in Eagle minimal essential medium (9, 10) supplemented with 10% human or calf serum. The cells were suspended in the same medium containing 5% calf serum before viral infection. Monolayer cultures of KB cells were used in some experiments under maintenance medium (infecting fluid) (13).

Viral stocks were prepared in suspension cultures. The derivation of the type 5 adenovirus wild-type (WT) strain and mutants has been reported (11; Ensinger, Ph.D. thesis). Stocks of temperature-sensitive mutants were tested for viral titer and reversion frequency before use. Virus was assayed by

means of a plaque assay (23) or an indirect immunofluorescence assay, as described previously (32, 44; Ensinger, Ph.D. thesis).

Preparation of antisera. The immunization of rabbits with purified virus, hexon, penton, and fiber has been described elsewhere (45, 46). The anti-hexon antiserum was prepared by immunizing rabbits with twice-recrystallized hexon protein that displayed only a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The specificity of the an-

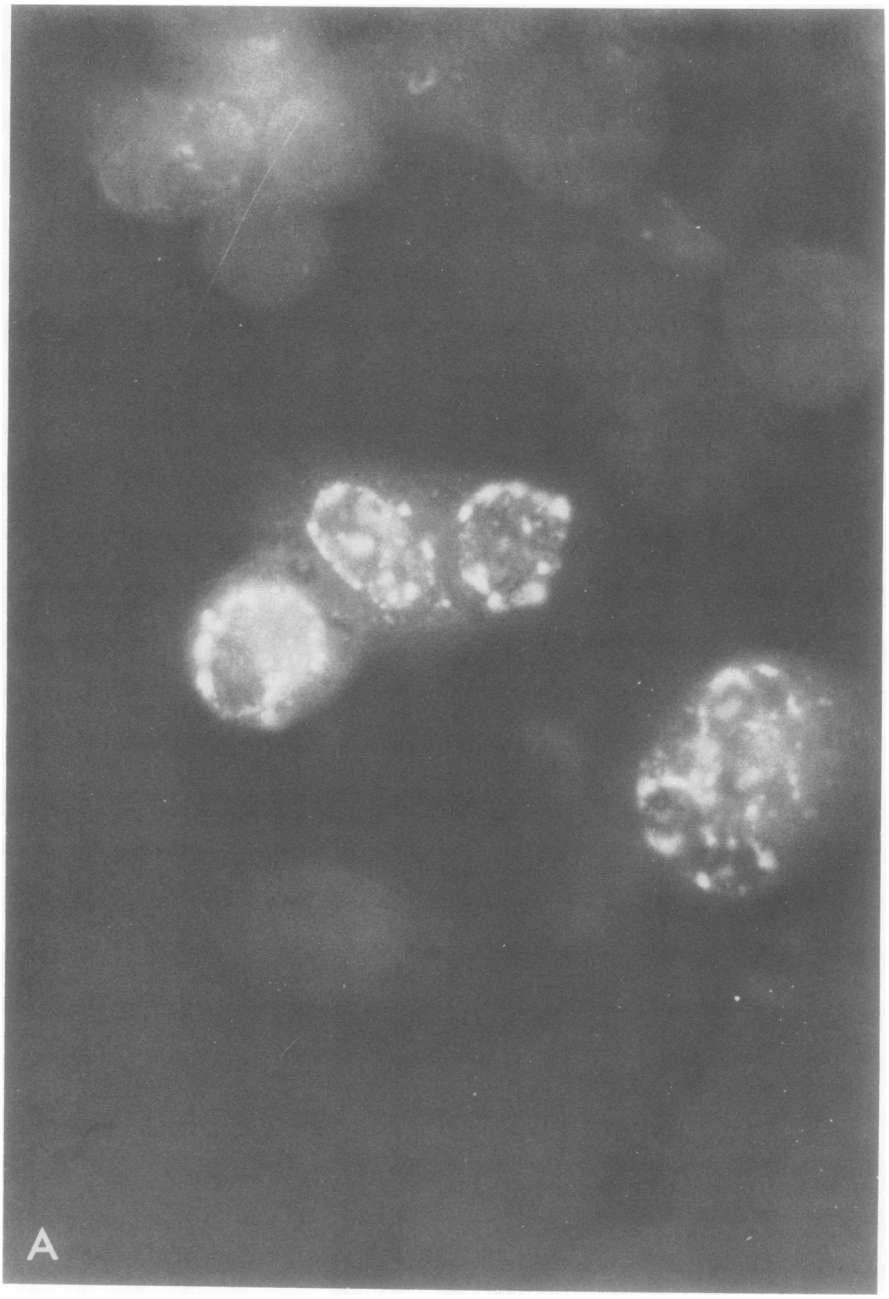
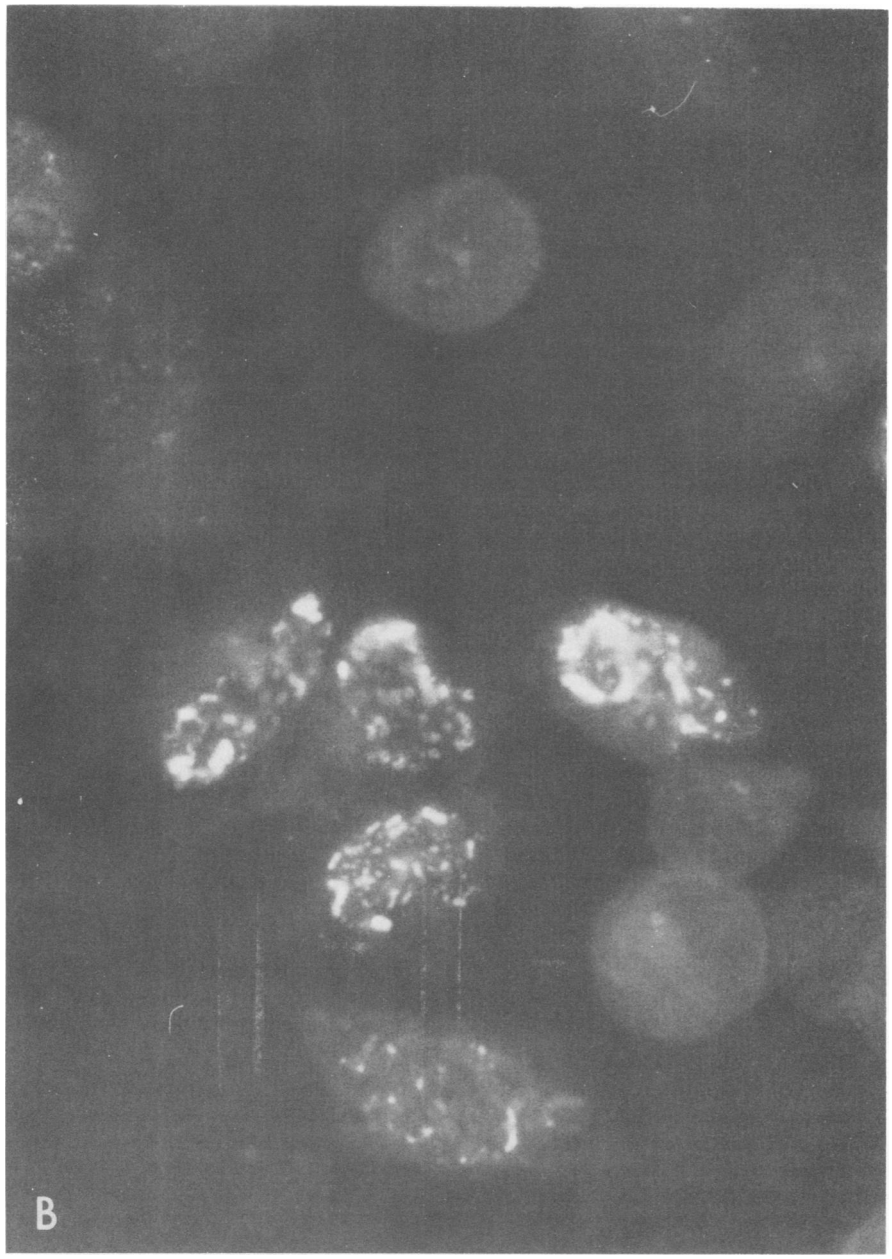


FIG. 2. *Indirect immunofluorescence of infected KB cell monolayers reacted with anti-fiber antiserum. Monolayers, grown on glass cover slips, were infected with WT and H5ts147 viruses and incubated at 39.5°C for 18 h before fixation in acetone. A two-step procedure employing specific rabbit anti-fiber antiserum and fluorescein-conjugated goat anti-rabbit serum was used. Stained monolayers were photographed under UV illumination. ×400. (A) H5ts147-infected cells; (B) WT-infected cells.*



tiserum for the hexon alone was ascertained by immunodiffusion and immunoprecipitation studies. The serum was shown to neutralize adenovirus and was specific for the trimeric hexon capsomer (36). Antiserum against the arginine-alanine-rich core protein was kindly supplied by L. Prage (Uppsala University).

Genetic studies. Complementation and genetic recombination experiments were performed as previously described (11).

Immunofluorescence. Monolayer cultures of KB cells were grown on glass cover slips in 35-mm plastic petri dishes. Cells were infected with 1 to 2 PFU of mutant or WT virus per cell. Adsorption was carried out for 3 h with shaking, followed by the addition of 3 ml of infecting fluid. Fixation was accomplished by using a procedure described earlier (5). After 18 h of incubation at 39.5°C or 40 to 48 h at 32°C, monolayers were washed with phosphate-buffered saline and air dried for 1 h at 37°C. The cover slips were fixed in absolute acetone at room temperature for 10 min and air dried an additional

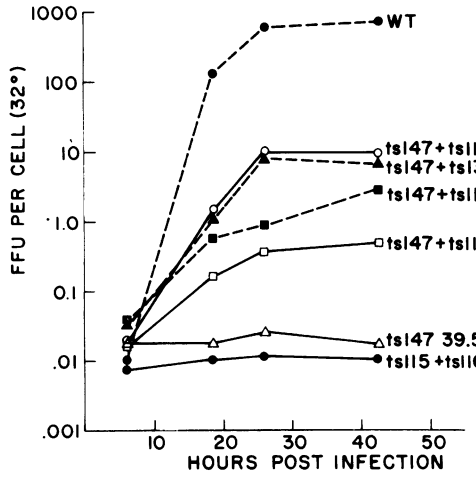


FIG. 3. *Complementation between H5ts147 and four hexon-minus temperature-sensitive mutants of type 5 adenovirus. Suspension cultures of KB cells were infected with 15 PFU of each mutant in pairs per cell, with one mutant alone or with WT virus, and incubated at 39.5°C. Samples were withdrawn from the 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.*

30 min. The indirect immunofluorescence assay was performed as described previously (32), using an appropriate dilution of a variety of specific antisera. Cover slips were inverted, mounted on glass microscope slides, and observed under UV illumination.

Multiplication cycles. Suspension cultures of KB cells at a density of 1×10^6 cells/ml were infected with 15 PFU of mutant or WT virus per cell, and adsorption was carried out for 2 h at either 32 or 39.5°C, depending on the experiment. The cells were centrifuged and resuspended in fresh prewarmed medium to a cell density of 2×10^5/ml and incubation was continued. 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 the fluorescent focus assay. Temperature shift was accomplished by transferring a Spinner culture from one water bath to another or by removing a portion of a culture to a fresh Spinner flask incubated at the desired temperature. The temperature equilibration time was 10 min.

Radioactive labeling and chase conditions. To label KB cells in suspension culture with [^{35}S]methionine, the cells were resuspended to a density of 2×10^6 to 4×10^6/ml in Eagle minimal essential medium lacking methionine. Cells were incubated for 15 min in the absence of this amino acid, whereupon [^{35}S]methionine (200 to 400 Ci/mmol) was added to a concentration of 20 to 80 μCi/ml in the presence of 1/20 the normal concentration of methionine. Chase of the radioactive label was accomplished by rapidly chilling, centrifuging, and resuspending the cells to a density of 2×10^5/ml in fresh prewarmed medium containing 5% calf serum and 1 mM unlabeled methionine. The effectiveness of the chase was documented by a constant level of trichloroacetic acid-precipitable radioactivity.

Cells were labeled with a ^{3}H-amino acid mixture in the same manner, except that Eagle minimal essential medium lacking seven amino acids was used; the medium was supplemented with normal concentrations of those amino acids not present in the labeling mixture. The concentration of label used was 2 to 10 μCi/ml. Chase of the radioactive label was accomplished in the same manner as described for methionine, except that excess unlabeled amino acids were not used.

Synthesis of viral capsomers. Experiments performed were similar to those reported earlier (46), except that cells were labeled for 15 min with a ^{3}H-amino acid mixture followed by a chase period of 30 min. Preparations of cell extracts and the sucrose gradient analysis were the same as described (46), except that the infected-cell preparations were placed on 10 to 40% linear sucrose gradients without dissociating agents and centrifuged for 7 h at 45,000 rpm at 20°C in a Spinco SW50.1 rotor or for 19 h at 30,000 rpm at 20°C in an SW41 rotor. Immunoprecipitation was performed by mixing an equal volume of a portion of a sucrose gradient fraction with an appropriate dilution of an antiserum prepared against purified hexon. The mixtures were incubated for 30 min at 37°C, after which an equal volume of goat anti-rabbit serum was added, and the mixture was incubated for an additional 30 min at 37°C. The reaction mixtures were centrifuged; the sedimented precipitates were washed three times in ice-cold phosphate-buffered saline, solubilized in 0.2 M NaOH, and counted directly in a toluene-based fluor containing Triton X-100. The proportion of the total radioactivity in each fraction precipitated by antiserum, minus the nonspecific precipitation in identical samples in which normal rabbit serum was substituted for the anti-hexon antiserum, was expressed as specific immunoprecipitation.

Viral purification. Virus was purified by resuspending the infected cells in 0.01 M phosphate buffer, pH 7.2, and sonically treating for 30 s in an MSE sonicator at maximum power. The disrupted cells were extracted three times with freon and then sedimented through CsCl of a density of 1.2 g/cm^3 onto a cushion of CsCl of a density of 1.4 g/cm^3. The viral band obtained was diluted with 0.01 M Tris buffer, pH 7.2, and sedimented to equilibrium in a preformed CsCl gradient of 1.2 to 1.4 g/cm^3. The virus was collected by puncturing the bottom of the tube and was stored at 4°C.

Polyacrylamide gel electrophoresis. Radioactively labeled protein samples were precipitated with trichloroacetic acid and washed with acetone; the precipitate was then suspended in gel sample buffer (1% SDS, 1% mercaptoethanol, 50 mM Tris, pH 6.8, and 10% glycerol) or dialyzed against sample buffer overnight at room temperature. Cell pellets were resuspended directly in this buffer by boiling for 1 min. Ten percent and 13% discontinuous polyacrylamide gels were prepared by using the buffer system

of Laemmli (22). The ratio of acrylamide to bisacrylamide was 30:0.8. Cylindrical gels 11 cm in length were electrophoresed at 3 mA/gel until the dye front was within 1 cm of the bottom of the tube. The gels were cut into 1-mm slices, solubilized in Protosol (New England Nuclear Corp.), and counted in toluene-Omnifluor (New England Nuclear Corp.) in a Packard scintillation spectrometer. Corrections were made for channel spillover when double-labeled samples were counted. Slab gels were pre-

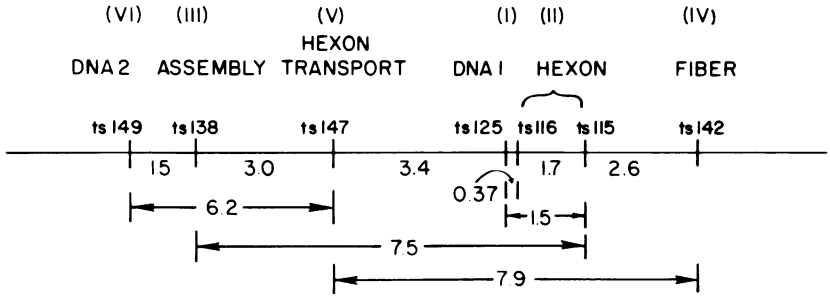


FIG. 4. *Genetic map of a portion of the type 5 adenovirus genome. The mean recombination frequencies derived from several two-factor crosses for each pair of mutants were plotted on a linear genetic map, according to the best additivities obtained. Selected mean recombination frequencies are shown. The left- and right-hand ends of the genetic map are oriented according to the physical map of adenovirus as determined by Grodzicker et al. (17). Roman numerals indicate the temporary complementation group designation (Ensinger, Ph.D. thesis).*

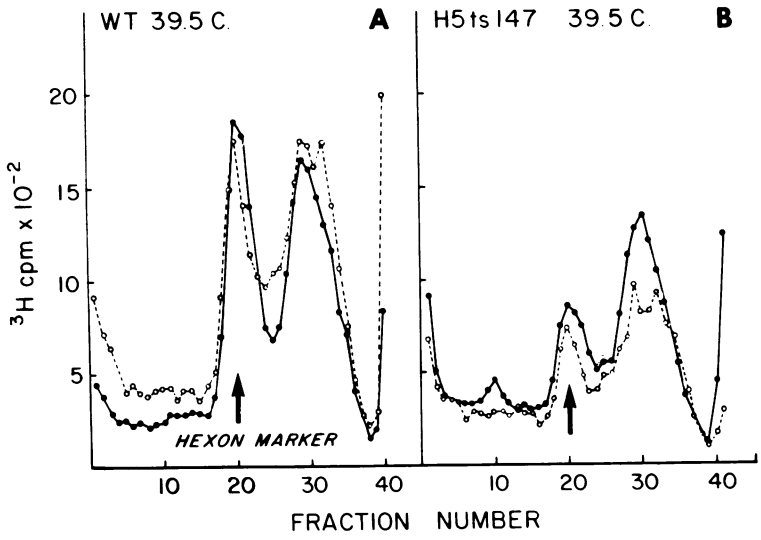


FIG. 5. *Synthesis of viral capsomers in infected cells at 39.5°C. Two suspension cultures of KB cells were infected with 100 PFU of WT and H5ts147 viruses per cell, respectively, and incubated at 39.5°C for 16 h. At that time the cultures were labeled for 15 min with 10 μCi of a ^{3}H-amino acid mixture per ml. One-half of each culture was harvested at the end of the pulse, whereas the remainder was resuspended in fresh prewarmed medium without label and incubated at 39.5°C for an additional 30 min. The cells were sedimented by centrifugation, washed once in ice-cold phosphate-buffered saline, and resuspended to a density of 1 × 10^7 cells/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 sonically treated for 30 s and then incubated for 1 h at 39.5°C. A 0.4-ml amount of each extract was layered onto a 10 to 40% (wt/vol) sucrose gradient and centrifuged for 7 h at 45,000 rpm in a Spinco SW50.1 rotor at 20°C. A ^{14}C-labeled hexon marker was used. (A) Extracts prepared from WT-infected cells; (B) extracts prepared from H5ts147-infected cells. Symbols: ○, cells harvested immediately after the 15-min pulse; ●, cells harvested after the 30-min chase. For ease of comparison, the patterns for the pulsed and chased samples obtained in separate gradients were superimposed on the same graph. Sedimentation was from right to left.*

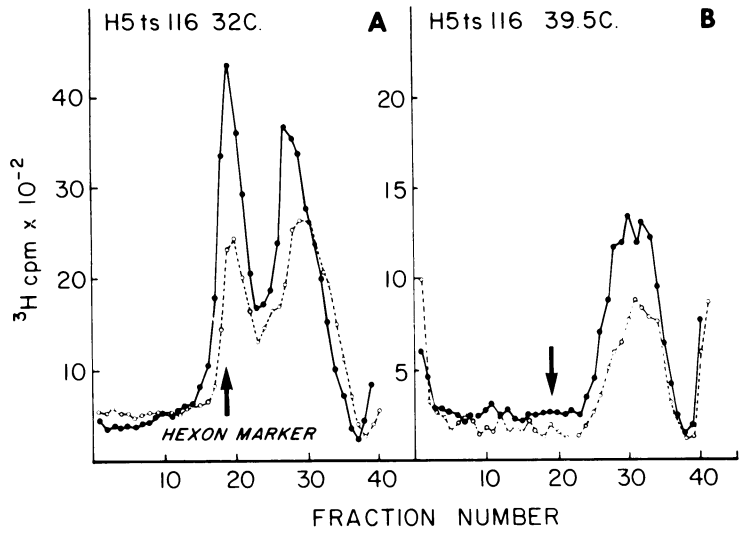


FIG. 6. *Synthesis of viral capsomers in cells infected with a hexon-minus mutant of type 5 adenovirus. Suspension cultures of KB cells infected with 100 PFU of H5ts116 virus per cell were incubated at 32 and 39.5°C. The cells were labeled for 15 min with a ^{3}H-amino acid mixture at 35 h postinfection at 32°C and 16 h postinfection at 39.5°C. One-half of each culture was harvested at the end of the 15-min pulse, and the remainder was harvested after a 30-min chase at the respective temperature. The preparation of extracts and centrifugation was the same as described in the legend to Fig. 5. A ^{14}C-labeled hexon marker was used. (A) H5ts116-infected cells at 32°C; (B) H5ts116-infected cells at 39.5°C. Symbols: ○, cells harvested after the 15-min pulse; ●, cells harvested after the 30-min chase. For ease of comparison, the patterns for the pulsed and chased samples obtained in separate gradients were superimposed on the same graph. Sedimentation was from right to left.*

pared in an apparatus described by Studier (37) with a 4% stacking gel. The samples containing no more than 10 μg of protein per well were electrophoresed at 70 V until the dye front reached the bottom of the gel. Gels were stained with Coomassie brilliant blue (Colab) and then dried under vacuum, and autoradiography was performed on Kodak Royal X-omat X-ray film.

Isotopes and chemicals. [^{35}S]methionine (200 to 400 Ci/mmol) was purchased from New England Nuclear Corp. ^{3}H-labeled algal protein hydrolysate (reconstituted) was purchased from Schwarz/Mann and New England Nuclear Corp. Fluorescein-conjugated goat anti-rabbit antiserum was obtained from Hyland Laboratories, and cycloheximide was purchased from Sigma Chemical Co.

RESULTS

Immunofluorescence. In the initial immunofluorescence screening procedure using anti-hexon antiserum and the temperature-sensitive mutants isolated in this laboratory, monolayer cultures of KB cells infected with H5ts147 at the nonpermissive temperature (39.5°C) were found to accumulate hexon antigen in the cytoplasm of the infected cells. Since the method of methanol fixation (42) was known to induce some distortion in the intracellular distribution of antigen, an alternate procedure using acetone as the fixative (5) was employed. Monolayers of KB cells, infected and fixed by the procedure described above, were studied, using a variety of specific antisera prepared against purified hexon, penton, fiber, and arginine-alanine-rich internal protein. In cells infected with H5ts147 and incubated at 39.5°C, immunologically reactive hexon accumulated solely in the cytoplasm (Fig. 1A), but in WT-infected cells fluorescence was confined to the nucleus (Fig. 1B). At 32°C, H5ts147-infected cells showed an intermediate hexon distribution with staining in both the nucleus and cytoplasm, whereas WT again showed hexon staining only in the nucleus (data not shown). In cells infected with WT virus or H5ts147 and maintained at 39.5°C, the distribution of fiber (Fig. 2), penton, and arginine-alanine-rich internal protein antigens were identical: all were found in the nucleus.

The defect in intracellular transport appeared to be specific for the hexon capsid protein, since other virion proteins were detected in their normal locations. Furthermore, since the anti-hexon antiserum used in these studies was reactive only against the native, trimeric capsomer and not the denatured polypeptide chains (36), it seemed likely that capsomers

were assembled into their native configuration in the cytoplasm of mutant-infected cells at the nonpermissive temperature. This result was in contrast to that obtained with those adenovirus mutants that did not produce immunologically reactive hexons in cells infected at 39.5°C (11) (termed "hexon-minus" mutants).

Genetic studies. The initial complementation studies performed on all available temperature-sensitive mutants suggested that H5ts147 was the single member of a nonoverlapping complementation group, notably distinct from the group of hexon-minus mutants that failed to produce immunologically detectable hexons at the nonpermissive temperature (11; Ensinger, Ph.D. thesis). To test the possibility that these findings resulted from intracistronic complementation (3) with the hexon-minus mutants and the subsequent spurious assignment of H5ts147 to a unique group, a series of complementation experiments was performed with a number of hexon-minus mutants that are members of the same complementation group. Recombination analysis indicated that the hexon-minus mutants occupy different loci within one cistron (11; Ensinger, Ph.D. thesis). The results of these experiments (Fig. 3) demonstrate significant complementation of H5ts147 with all the hexon-minus mutants tested, making the occurrence of intracistronic complementation unlikely.

Recombination studies using pairwise crosses of temperature-sensitive mutants, however, provided a more precise genetic analysis. A reasonably additive genetic map of a portion of the adenovirus genome has been constructed using two-factor crosses. The genetic map in Fig. 4 was derived from the results of three to five independent crosses for each pair of mutants. Map positions were assigned (using the mean recombination frequencies) on the basis of the best additivities obtained and the complementation group assignments in the case of some closely linked genes. Although the recombination frequencies varied from one experiment to another, the relative map order always remained the same. These data unambiguously place H5ts147 at a unique position on the genetic map, separated from the group of hexon-minus mutants by at least one cistron, that of H5ts125, a mutant defective in DNA synthesis (11).

The results of these experiments are evidence of the nonidentity of hexon transport and hexon-minus mutants. These data also imply that two unique viral gene products are essential for the formation of the hexon structural protein and for the transport of the hexon into the nucleus.

Synthesis of viral capsomers. It may be inferred from the immunofluorescence experiments described that trimeric hexon capsomers were formed in the cytoplasm of H5ts147-infected cells cultured at 39.5°C. To ascertain the validity of this conclusion, velocity sedimentation in sucrose gradients of radioactively labeled, infected-cell extracts was carried out as described above. At 15 h after infection, during the period of maximum viral protein synthesis at 39.5°C, cells were labeled with ^{3}H-amino acids for 15 min and then chased at 39.5°C for 30 min in nonradioactive medium prior to the preparation of extracts. Similar experiments were performed in parallel at 32°C. The results from cells infected with WT virus, H5ts147, and H5ts116 (a hexon-minus mutant) are shown in Fig. 5 and 6. The findings for the WT-infected culture (Fig. 5A) are similar to those reported earlier (46). In H5ts147-infected cultures (Fig. 5B), 12*S* hexons were clearly produced at 39.5°C, although in reduced amounts when compared with WT; at 32°C relatively more 12*S* hexons were produced than at 39.5°C. In contrast, detectable 12*S* hexon capsomers were not made in H5ts116-infected cells at the nonpermissive temperature (Fig. 6B), which was consistent with the failure to detect hexons with anti-hexon antiserum in immunofluorescence studies, and attests to the specificity of the antiserum employed. At 32°C trimeric hexons were formed in H5ts116-infected cells (Fig. 6A).

In those gradients in which samples were harvested and centrifuged immediately after the labeling period and before a chase, material was seen with an approximate sedimentation coefficient of 3.4*S* (Svedberg unit) near the top of the gradient (46). This protein represents unassembled polypeptide chains of the viral capsomers (46) and was converted into the more rapidly sedimenting capsomers in both WT- and H5ts147-infected cultures during the chase pe-

TABLE 1. *Immunoprecipitation of peak fractions of the 12S (hexon) region from sucrose gradients*

Determination	Total cpm	cpm in precipitate[a]	cpm in precipitate (%) (%)	Specific precipitation (%)[b]
WT	914	782	86	70
H5ts147	392	342	87	68

[a] Immune coprecipitation employing specific anti-hexon antiserum was performed on samples of the peak fraction from the 12*S* region of sucrose gradients.

[b] Specific precipitation was calculated from the total precipitation by subtraction of nonspecific trapping in samples in which normal rabbit serum was substituted for anti-hexon antiserum.

riod at 39.5°C. In H5ts116-infected cells chased at 39.5°C, during which time no 12S hexons were formed, labeled protein remained at the top of the gradient (Fig. 6B) and presumably represented nascent hexon polypeptide chains that were unable to form capsomers at the nonpermissive temperature. In all cases, greater than 75% of the trichloroacetic acid-precipitable radioactivity added was recovered from the gradient.

Fractions taken from sucrose gradients in the 12S region of the gradient were subjected to immunoprecipitation, using the same anti-hexon antiserum as that used in the immunofluorescence studies. The results of the specific immunoprecipitation of the peak fractions of the 12S region of the gradients are given in Table 1. Hexons from WT- and H5ts147-infected cells labeled at the nonpermissive temperature were precipitated to a similar extent, which further suggests that hexons of native configuration were synthesized at 39.5°C in H5ts147-infected cells.

One-step growth curves after temperature shift. Efforts to study the functional effects of this temperature-sensitive lesion on viral replication were directed toward determining the effect of temperature shift on infectious viral productions at late times in the growth cycle. To enquire whether the ts gene product synthesized at 39.5°C could renature and function at 32°C, 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. The results (Fig. 7 and 8) show that neither shift-up nor shift-down alone had any effect on viral production in WT-infected cultures, except for the expected alteration in the rate of viral replication owing to the temperature change. Inhibition of protein synthesis at the time of step-up or step-down in WT-infected cultures arrested viral production after a 1- to 3-h lag, depending on the temperature. During that time period, however, viral production proceeded normally. In H5ts147-infected cultures, shift-up to the nonpermissive temperature (Fig. 7) resulted in the arrest of viral production within 1 to 2 h; cycloheximide did not greatly increase the inhibition. The kinetics of inhibition were similar to the WT-infected culture treated with cycloheximide at the time of shift-up.

Shift-down of mutant-infected cells to 32°C (Fig. 8) was followed by the initiation of viral production after a delay of 2 to 6 h, depending

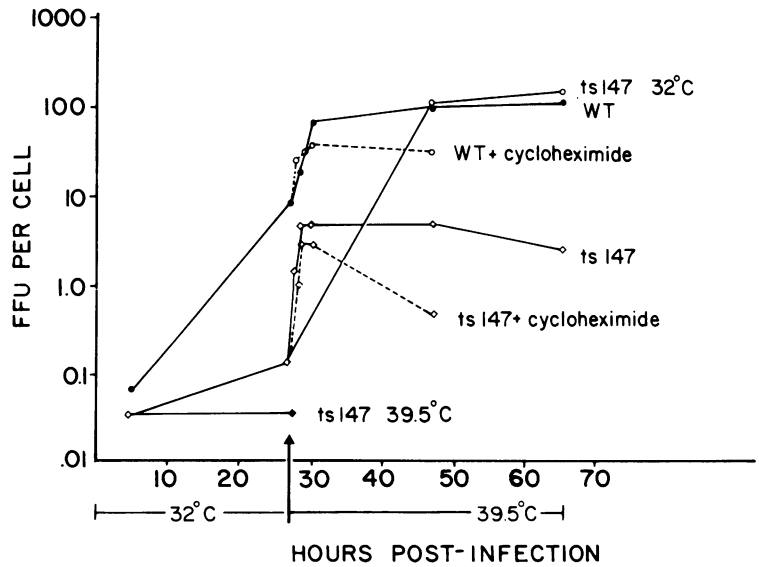


FIG. 7. *Viral multiplication after shift-up to 39.5°C. Suspension cultures of KB cells were infected with 15 PFU of either WT or H5ts147 virus per cell and incubated at 32°C. At 27 h postinfection, portions of each culture were placed in fresh Spinner flasks and incubated at 39.5°C. A WT- and H5ts147-infected culture were each treated with 10 μg of cycloheximide per ml at the time of the temperature shift. Control cultures infected with H5ts147 were incubated at 32 and 39.5°C, respectively, for the entire experiment. Samples were withdrawn at various times, frozen and thawed to release intracellular virus, and titrated for infectivity at 32°C using the fluorescent focus assay. Symbols: —○—, H5ts147, 32°C control; ◆, H5ts147, 39.5°C control; ●, WT shift-up; -----○-----, WT shift-up plus cycloheximide; —◇—, H5ts147 shift-up; -----◇-----, H5ts147 shift-up plus cycloheximide. FFU, Focus-forming units.*

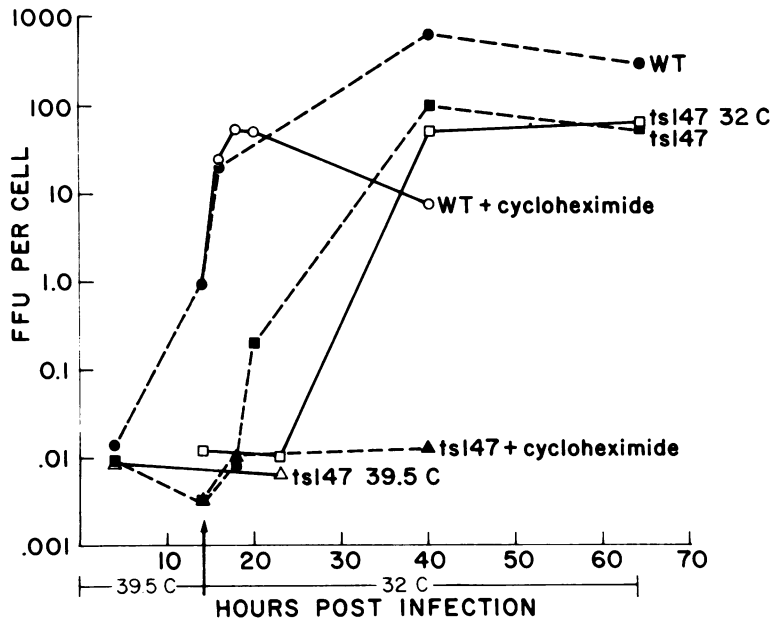


FIG. 8. *Viral multiplication after shift-down to 32°C. Suspension cultures of KB cells were infected with 15 PFU of either WT or H5ts147 virus per cell and incubated at 39.5°C. At 14 h postinfection, portions of each culture were placed in fresh Spinner flasks and incubated at 32°C. A WT- and H5ts147-infected culture were each treated with 10 μg of cycloheximide per ml at the time of the temperature shift. Control cultures infected with H5ts147 were incubated at 32 and 39.5°C, respectively, for the duration of the experiment. Samples were withdrawn at various times, frozen and thawed to release intracellular virus, and titrated for infectivity at 32°C using the fluorescent focus assay. Symbols: □, H5ts147, 32°C control; △, H5ts147, 39.5°C control; ●, WT shift-down; ○, WT shift-down plus cycloheximide; ■, H5ts147 shift-down; ▲, H5ts147 shift-down plus cycloheximide. FFU, Focus-forming units.*

upon the time at 39.5°C before temperature shift: the longer the time at 39.5°C, the shorter the delay. The rate of viral production almost reached that of WT-infected cultures shifted to 32°C, and the final titer attained was equal to that of H5ts147-infected cells maintained at 32°C for the duration of the experiment. The addition of cycloheximide to H5ts147-infected cultures at the time of temperature shift-down completely inhibited the subsequent initiation of viral production as well as hexon transport measured by immunofluorescence. Therefore, the formation of infectious virions in H5ts147-infected cultures after a shift to 32°C required continuing protein synthesis, even though a considerable quantity of hexons was present in the cytoplasm of these cells and all other virion structural proteins were in the nucleus at the time of change to the permissive temperature.

Transport of hexons after shift to 32°C. Since immunologically reactive 12*S* hexon capsomers were made in the cytoplasm of H5ts147-infected cells at 39.5°C and infectious viral production demanded de novo protein synthesis after shift-down, experiments were devised to determine whether the hexon capsomers produced at 39.5°C could subsequently be transported into the nucleus and assembled into virions in a conservative manner after shift-down to 32°C. The leakiness of nuclei from adenovirus-infected cells late in infection (45) makes direct measurement of the quantity of hexon protein in the nucleus difficult to interpret.

Cells infected and maintained at 39.5°C until 14 h after infection, a time when late viral proteins were being synthesized, were labeled for 2 h at 39.5°C with [^{35}S]methionine, as described above. The radioactive label was "chased" in unlabeled medium for 30 min at 39.5°C before the cultures were shifted to 32°C. This chase period served the double purpose of depleting the cellular pool of radioactive amino acids available for protein synthesis and allowing all the unassembled polypeptide chains to be completed and converted to capsomers before the temperature was lowered. After shift-down, the cultures were incubated at 32°C for 24 h to allow the maximum production of virions. The virus was then purified from these cells, and purified virions were disrupted and electrophoresed on cylindrical SDS-polyacrylamide gels. The proportion of radioactively labeled hexons

recovered from the virion in WT- and H5ts147-infected cells was compared by weighing the appropriate areas under the curves formed from plots of the radioactivity of the virion polypeptides.

Although viral production in H5ts147-infected cells was less prodigious than in cells infected with WT virus and total recovery of both radioactivity and viral particles was lower in the case of H5ts147, both parameters were decreased roughly in parallel, and the specific activities of virions recovered from WT- and H5ts147-infected cultures were approximately the same. The analysis on polyacrylamide gels shown in Fig. 9 indicates that a similar proportion of the total radioactivity was present in hexons and other virion polypeptides recovered from WT and H5ts147 virus after the long chase period. Therefore, hexons that accumulated in the cytoplasm at 39.5°C could be efficiently used in viral assembly at 32°C. The absolute recovery of radioactivity applied to the gels was the same. If virions were purified from infected cells labeled before the onset of viral capsid protein synthesis and then subjected to chase periods at 39.5 and 32°C as described, the virion proteins contained less than 1% of the level of radioactive polypeptides than those from cultures labeled during the period of capsid protein synthesis. These control experiments demonstrated that protein degradation and reutilization of isotope in protein synthesis during the chase period could not account for this level of radioactivity found in virion polypeptides (Fig. 9).

Synthesis of polypeptides in infected cells. The polypeptide products of genes containing missense mutations do not have the proper conformation at the restrictive temperature; therefore, the defective proteins may be degraded after their synthesis and fail to accumulate in the normal manner (16, 34, 41). In the absence of other criteria, it was considered that this property might be exploited to identify tentatively the gene product affected by the H5ts147 mutation. For this purpose, SDS-polyacrylamide gel analyses of [^{35}S]methionine-labeled, infected KB cells were carried out. Infected cells were incubated at 39.5°C for 17 h, after which they were labeled for 15 min with [^{35}S]methionine and then chased at 39.5°C in nonradioactive medium containing a 10-fold excess of unlabeled methionine. Samples were withdrawn at various times during the chase

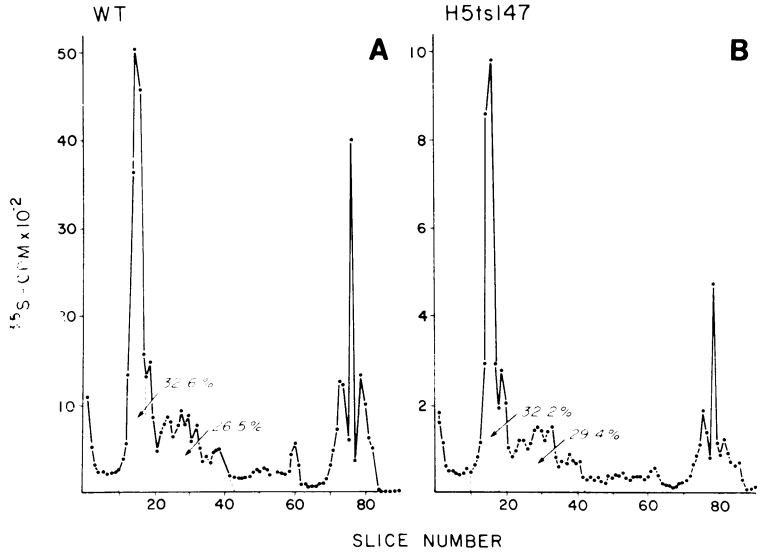


FIG. 9. *Virion assembly of polypeptides labeled at 39.5°C after shift-down to 32°C. Suspension cultures of KB cells were infected with 100 PFU of WT or H5ts147 virus per cell and incubated for 14 h at 39.5°C. The cells were then labeled with [^{35}S]methionine for 2 h at 39.5°C, as described in the text. After a 30-min chase period at 39.5°C, the cultures were shifted to a 32°C water bath and incubation was continued for an additional 26 h. The cells were harvested by centrifugation, and virions were purified as described in the text. Purified virions were dialyzed overnight at room temperature against gel sample buffer containing 2% SDS, 1% β-mercaptoethanol, and 0.0625 M Tris-hydrochloride buffer, pH 6.8. After dialysis, 10% glycerol was added to each sample, and the disrupted virions were subjected to electrophoresis at 3 mA/gel on cylindrical 10% SDS-polyacrylamide gels. The gels were then sliced, solubilized, and counted. The proportion of isotope in virion hexons and other capsid protein is noted. The direction of migration was from left to right. (A) Virions from WT-infected cells; (B) virions from H5ts147-infected cells.*

period, and the cell pellets were resuspended in gel sample buffer containing SDS and boiled for 1 min. Samples were electrophoresed on SDS-polyacrylamide slab gels and analyzed by autoradiography. The intensities of the labeled polypeptides were compared in WT-, H5ts147-, and mock-infected cells.

As shown in Fig. 10, the overall pattern of labeled polypeptides, which was similar to published studies (1, 21), was the same in WT- and H5ts147-infected cells. The relative amounts of labeled hexon polypeptides (II) in the samples from WT- and H5ts147-infected cells were similar, although the total incorporation of radioactive label into proteins in the mutant-infected cultures was about half that of the WT. During the chase period, P-VII, the precursor to protein VII (1), remained stable in H5ts147-infected cultures, but protein VII was not detectable during the chase. This result is consistent with the evidence that the conversion of P-VII to VIII is a late step in viral assembly (1, 21), since neither infectious virus nor viral particles were produced in H5ts147-infected cultures at 39.5°C (unpublished data). Similarly, polypeptides VI and VIII were not formed from their apparent precursors, the P-VI (27K) and P-VIII (26K) polypeptides (1, 21, 31). However, there was a rapid degradation of the P-VI polypeptide in H5ts147-infected cells, whereas P-VIII remained stable. In WT-infected cells, the intensity of the P-VI polypeptide also decreased during the chase, but the decrease was accompanied by a concomitant appearance of virion pro-

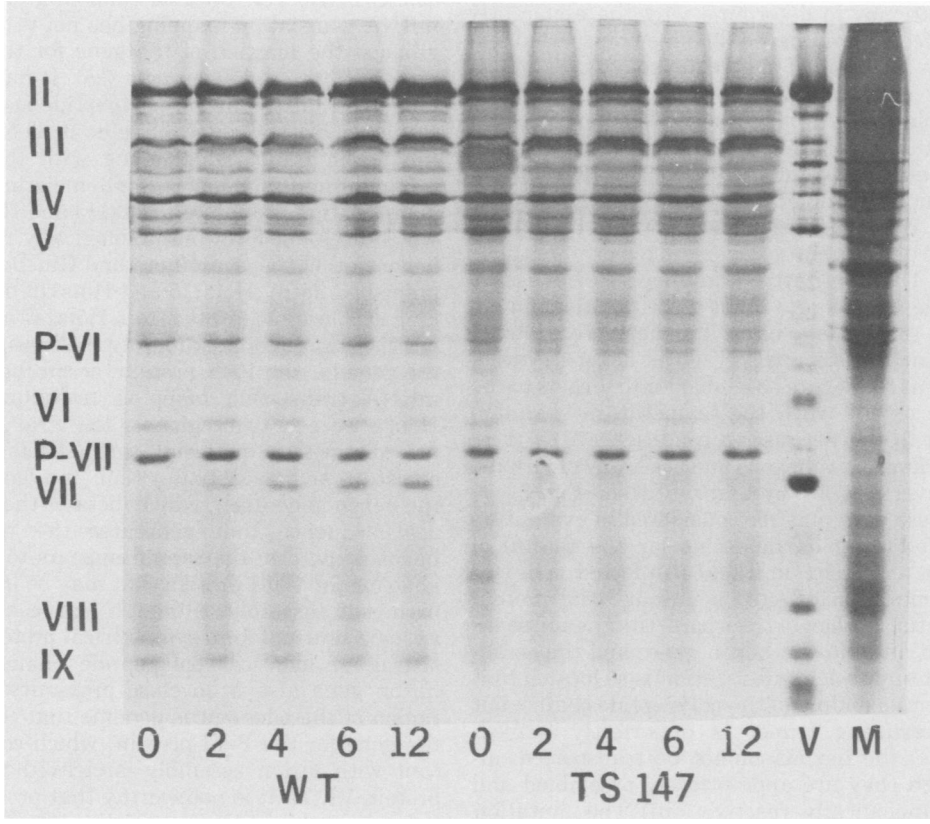


FIG. 10. *SDS-polyacrylamide gel autoradiogram of [^{35}S]methionine-labeled infected-cell extracts from WT- and H5ts147-infected cells pulse labeled and chased at 39.5°C. KB cells in suspension culture were infected with 100 PFU of WT or H5ts147 virus per cell at 39.5°C and incubated for 17 h. The cells were then labeled for 15 min with 80 μCi of [^{35}S]methionine per ml and chased for up to 12 h at 39.5°C in complete medium containing a 10-fold excess of unlabeled methionine (10 mM). Samples were withdrawn from the cultures immediately after the pulse label and at the indicated times during the chase. The washed cell pellets were disrupted in gel sample buffer (2% SDS, 1% β-mercaptoethanol, 0.0625 M Tris-hydrochloride, pH 6.8, and 10% glycerol) and boiled for 1 min. Electrophoresis was carried out at 80 V on a 13% SDS-polyacrylamide slab gel. V, Purified virion; M, mock-infected cells labeled for 15 min and chased for 1 h. The numbers under the other sample slots represent the time of chase before each sample was harvested from the corresponding WT- or H5ts147-infected culture.*

tein VI. All other polypeptides in the H5ts147-infected cells, including hexon, appeared to remain stable during the chase period. At 32°C P-VI was synthesized and processed to VI at a rate similar to that in WT-infected cells, with no additional instability detectable (data not shown).

DISCUSSION

H5ts147, a conditionally lethal, temperature-sensitive mutant of type 5 adenovirus, does not assemble infectious virions or empty capsids at the nonpermissive temperature (39.5°C), although all of the major capsid proteins are formed into immunologically reactive and structurally intact multimeric proteins. The intact hexons, however, are not transported in the nucleus. Hence, this mutant was studied in an attempt to determine why the apparently unaffected protein could not be transported and to reveal some of the requirements for movement of a protein into the nucleus from its cytoplasmic site of synthesis. The hexons of the mutant, which assemble normally but accumulate aberrantly in the cytoplasm at the nonpermissive temperature, are indistinguishable from WT virus hexons according to the following criteria: immunofluorescence examinations (Fig. 1), quantitative immunoprecipitin assays (Table 1), and rate zonal centrifugations in sucrose gradients (Fig. 5). Furthermore, H5ts147 mutant hexons and WT virus hexons synthesized at 39.5°C are assembled into virions to the same extent when the temperature is shifted down to the permissive condition (32°C) (Fig. 9). Transport of hexons and assembly of virions, however, require protein synthesis (Fig. 8).

These data must be considered in evaluating the following explanations for the failure of hexon transport in H5ts147-infected cells. (i) The mutation affected a unique viral protein essential for hexon transport. (ii) The missense mutation is in the hexon gene, and the consequent amino acid substitution is so located that it permits folding of the polypeptide chains, but the resulting trimer is imperfectly formed. Hence, the hexons cannot be transported, although they are appropriately assembled and immunologically reactive. (iii) The mutation produced a defective protein required for hexon assembly, and faulty assembly does not permit effective transport.

Concordant with the findings that in H5ts147-infected cells the hexons made at 39.5°C are indistinguishable from WT hexons and can be assembled into virions, the latter hypothesis seems the least likely. Two other findings bear upon the conclusion that the ts147 mutation is not in a gene coding for a distinct protein needed for hexon assembly: (i) H5ts2 (49, 51), which is in the same complementation group as H5ts147 (Kauffman, Williams, and Ginsberg, unpublished data), appears to be a mutation in the region of the hexon gene, according to interpretation of data obtained by physical mapping (17, 29, 50), and (ii) specific mRNA translation mapping (25) relates the hexon gene to the same region of the genome proposed as the locus for the H5ts147 (Fig. 1) and H5ts2 alleles. However, the allocation of the ts147 and ts2 mutations to the hexon gene per se is inconsistent with the finding that new protein synthesis is required for hexon transport in H5ts147-infected cells. Moreover, the precise assignment of the mutation is complicated by the findings that the precursor for protein VI, termed P-VI protein, is degraded at 39.5°C in H5ts147-infected cells (Fig. 10), and mRNA translation mapping has not yet distinguished the location of the gene for the P-VI protein from the hexon gene (25). It must also be noted that the hexon polypeptide is not unstable in H5ts147-infected cells at 39.5°C, but the hexon is degraded, along with the P-VI protein in mutants belonging to a distinct complementation group (i.e., H5ts115 and H5ts116) which does not form immunologically reactive hexons at 39.5°C (Kauffman and Ginsberg, unpublished data). H5ts115 and H5ts116 mutants map at a locus separated from H5ts147 and at a position that is approximately that assigned to the gene for the 100K protein, according to the mRNA translation mapping technique (25). Hence, at least two regions of the viral genome provide genetic material that affects hexon structure and possibly function: one consists of the hexon gene itself, which dictates the amino acid sequence that generates the trimeric hexon structure; the second appears to be the gene for the 100K protein that may be involved in hexon assembly, although this conclusion appears unusual for a symmetrical protein consisting of identical polypeptide chains. The hexon gene also is in close proximity to the region of the adenovirus genome that contains the gene for the P-VI protein, which concomitant with virion assembly is cleaved to form protein VI (1). It is noteworthy that protein VI is associated with hexons in the intact virion (13). Further studies are required to provide data that P-VI may serve a critical function in hexon transport, that there is a stringent requirement for precise hexon assembly to permit hexon transport, and that the 100K protein plays a central role in hexon morphogenesis.

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