JOURNAL OF VIROLOGY, Nov. 1981, p. 491-500
0022-538X/81/110491-10$02.00/0

Vol. 40, No. 2

Characterization of Two Temperature-Sensitive Mutants of Type 5 Adenovirus with Mutations in the 100,000-Dalton Protein Gene

ELIZABETH A. OOSTEROM-DRAGON† AND HAROLD S. GINSBERG*

Department of Microbiology and Cancer Center, Columbia University College of Physicians and Surgeons, New York, New York 10032

Received 18 February 1981/Accepted 7 July 1981

Complementation analysis assigned the mutations of strains H5*ts*115 and H5*ts*116, two hexon-minus mutants, to the 100,000-dalton (100K) protein gene. Heterotypic marker rescue (i.e., type 5 adenovirus [Ad5] temperature-sensitive mutants DNA × *Eco*RI restriction fragments of Ad2 DNA) confirmed the results of previous marker rescue mapping studies, and the heterotypic recombinants yielded unique hybrid (Ad5-Ad2) 100K proteins which were intermediate in size between Ad5 and Ad2 proteins and appeared to be as functionally active as the wild-type 100K protein. Phenotypic characterization of these mutants showed that both the hexon polypeptides and the 100K polypeptides were unstable at the nonpermissive temperature, whereas fiber and penton were not degraded, and that the 100K protein made at 39.5°C could not be utilized after a shift to the permissive temperature (32°C). The role of the 100K protein in the assembly of the hexon trimer was also examined by in vitro protein synthesis. Normally, hexon polypeptides synthesized during an in vitro reaction are assembled into immunoreactive hexons. However, this assembly was inhibited by preincubation of the cell extract with anti-100K immunoglobulin G; neither anti-fiber immunoglobulin G nor normal rabbit immunoglobulin G inhibited hexon assembly. It is postulated that an interaction between the 100K protein and hexon polypeptides is required for effective assembly of hexon trimers.

The isolation and characterization of conditionally lethal, temperature-sensitive (*ts*) mutants of type 5 adenovirus (Ad5) (6, 29, 45) have revealed many mutants with mutations affecting the structure and function of hexons (the major capsid protein). Initially, these mutants were classified into a number of complementation groups, but collaborative studies have suggested that they should be partitioned into only two nonoverlapping complementation groups (11). Classical recombination analyses (6, 16, 46) and heterotypic recombinations (Ad5*ts* × Ad2*ts*) followed by analyses of restriction fragment patterns (13, 14, 22, 30, 43) have clearly indicated that the mutations of these two groups of mutants occur in noncontiguous regions of the genome. Marker rescue studies have confirmed these findings (2, 9). Moreover, in vitro translation of mRNA's selected by hybridization with specific DNA fragments (21, 22, 24, 28) and DNA-RNA hybrid arrest of in vitro mRNA translation (24, 26) have demonstrated that the hexon gene, which is affected in one set of mutants, is located between 50 and 60 map units on the viral genome, whereas the mutations of the other mutants are situated between about 65 and 80 map units. This latter region encodes a late, nonstructural, 100,000-dalton viral protein (100K protein), a nonstructural 33K protein, and the pVIII protein, whose processed product (protein VIII) is associated closely with hexons in virion capsids (1, 7, 8).

The studies reported here were initiated to determine whether the mutations of strains H5*ts*115 and H5*ts*116, which were mapped by heterotypic recombination between 65 and 80 map units (11), were mutations involving the 100K protein gene or the neighboring gene for the pVIII protein. In this paper we show that H5*ts*115 and H5*ts*116 are results of mutations in the gene encoding the 100K protein. In addition, we characterize the phenotypes of these mutants and suggest that the 100K protein, which is essential for hexon maturation, may serve as a "scaffolding" protein.

MATERIALS AND METHODS

Cells and virus. KB cells were grown in a suspension culture in Eagle minimal essential medium

† Present address: Department of Biology, Brookhaven National Laboratory, Upton, NY 11973.

(GIBCO Laboratories) supplemented with 10% horse or human serum. Before infection with adenovirus, the cells were suspended in the same medium containing 5% calf serum. Monolayer cultures of KB cells under maintenance medium (infecting fluid) were used in some experiments (10). HeLa cells were grown in monolayer cultures in Eagle basal medium (GIBCO) supplemented with 10% calf serum.

The derivations of the Ad5 wild-type strain and mutants which we used have been described previously (6). Ad2$^+$ND1*ts*4 was kindly provided by T. Grodzicker. Plaque-purified stocks of *ts* mutants were tested for viral titers and reversion frequencies before use. A plaque assay (44) or an indirect immunofluorescence focus assay (27, 36) was used to quantitate viral infectivity.

Preparation of virus. Ad5 and Ad2 were purified as previously described (19).

Immunofluorescence. Indirect immunofluorescence procedures were performed as described previously (25).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18) in a slab gel apparatus, as described by Studier (33).

In vitro synthesis. The in vitro system of Wilhelm et al. (42) was used. Cytoplasmic extracts of wild-type Ad5-infected KB cells were prepared by Dounce homogenization. Nuclei and unbroken cells were removed by two cycles of centrifugation at 1,000 × *g* for 10 min; the supernatants were adjusted to 0.02 M Tris-hydrochloride (pH 7.6), 0.005 M magnesium acetate, and 0.08 M KCl and then applied to a Sephadex G-25 column (15 by 25 cm). The column was eluted with a solution containing 0.02 M Tris-hydrochloride (pH 7.6), 0.005 M magnesium acetate, 0.08 M KCl, and 0.006 M β-mercaptoethanol. The material in the void volume was collected and adjusted to a concentration of 40 units of absorbance at 260 nm per ml. For amino acid incorporation studies, reaction mixtures contained 0.6 volume of cytoplasmic extract, 0.50 M Tris-hydrochloride (pH 7.6), 0.0035 M magnesium acetate, 0.148 M KCl, 0.001 M dithiothreitol, 0.001 M ATP, 0.002 M GTP, 0.0066 M creatine phosphate, 100 μg of phosphocreatine kinase per ml, and 2.5 μCi of mixed ^{3}H-amino acids per ml.

Immunoprecipitation. Immunoprecipitation reactions were performed as previously described (25).

RESULTS

Genetic studies. In previous studies (6, 16; P. Luciw and H. S. Ginsberg, manuscript in preparation), it was shown that two sets of *ts* mutations which affect hexons map at noncontiguous regions of the genome. One group, containing the mutations in strains H5*ts*115 and H5*ts*116, appeared by genetic recombination to map in the region assigned to the gene for the 100K protein. Ad2$^+$ND1*ts*4, a strain with a mutation which has been mapped physically within the coordinates 69 to 71 on the adenovirus genome (30), has phenotypic characteristics similar to those of H5*ts*115 and H5*ts*116 (11). Therefore, complementation studies were performed (6) to determine whether the Ad5 *ts* mutants were in the same complementation group as Ad2$^+$ND1*ts*4. Table 1 shows that neither H5*ts*115 nor H5*ts*116 complemented Ad2$^+$ND1*ts*4. H5*ts*142, a strain with a mutation in the fiber gene (6; C. C. Cheng and H. S. Ginsberg, manuscript in preparation), was used as a control for positive complementation. These complementation data indicated that H5*ts*115 and H5*ts*116 also have mutations in the 100K protein gene. It should also be noted that H5*ts*115 and H5*ts*116 complement strains with mutations in the hexon gene (11, 16).

Analysis of *ts*$^+$ heterotypic recombinants. Heterotypic marker rescue of H5*ts*116 with Ad2 *Eco*RI-B fragments yielded *ts*$^+$ viruses whose DNAs showed that a recombination event had occurred between coordinates 59.5 and 70.7 when the DNAs were examined by restriction endonuclease digestion (unpublished data). These findings implied that the functional 100K proteins produced by these recombinants were either hybrids or Ad2 proteins. Since the Ad2 100K protein migrates faster than the related Ad5 protein during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (i.e., it is slightly smaller or differs in phosphorylation), it was possible to determine whether the recombinants expressed the Ad2 100K protein or an intertypic hybrid protein. This analysis was performed by using sodium dodecyl sulfate-polyacrylamide gels to electrophorese the polypeptides synthesized in KB cells infected with each of the recombinants, H5*ts*116, and Ad2 wild-type virus. The 100K polypeptides of the host recombinants had mobilities that were intermediate between those of the parental 100K polypeptides; all of the other viral polypeptides had characteristics of Ad5 proteins (Fig. 1). These data confirmed the genetic evidence that the mutations of H5*ts*116 and H5*ts*115 were in the gene encoding the 100K protein, and in addition they demonstrated the

TABLE 1. *Complementation between ts mutants*[a]

Mutant	Complementation index			
	H5*ts*116	H5*ts*115	H5*ts*142	Ad2$^+$ND1*ts*4
H5*ts*116	(0.004)[b]			
H5*ts*115	0.67	(0.091)		
H5*ts*142	796		(0.083)	
Ad2$^+$ND1*ts*4	0.192	0.792	310	(0.048)

[a] The degree of complementation is expressed as the complementation index (CI), which was determined by the following formula: CI = (yield per cell of mixed infection)/(sum of yields of single infections).

[b] The values in parentheses indicate the yields of single infections, expressed as focus-forming units per cell.

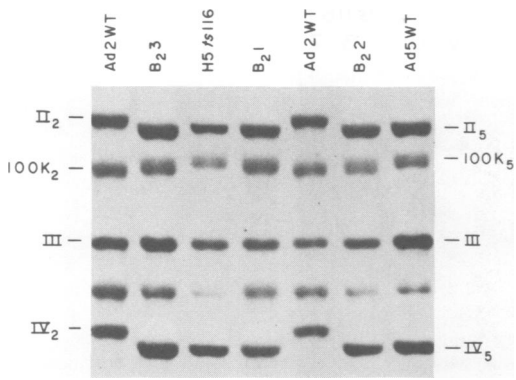

FIG. 1. *Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel containing [^{35}S]methionine-labeled polypeptides from lysates of cells infected with H5ts116, wild-type Ad2 (Ad2WT), and intertypic recombinants generated by marker rescue. KB monolayers were infected at a multiplicity of approximately 20 PFU/cell with wild-type Ad2, H5ts116, and the three intertypic recombinants (B_21, B_22, and B_23) produced by marker rescue crosses between H5ts116 and Ad2 EcoRI B fragments B_21, B_22, and B_23; then the monolayers were incubated at 39.5°C. At 16 h postinfection, the medium was removed from each dish and replaced with methionine-depleted minimal essential medium containing 50 μCi of [^{35}S]methionine per ml. The samples were pulse-labeled for 1 h at 39.5°C. The cells were scraped from the dishes, washed once with phosphate-buffered saline, and suspended in gel sample buffer at a concentration of 10^6 cells per ml. The samples were sonicated, boiled for 2 min, applied to a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, and subjected to electrophoresis for 3.5 h at 100 V. Ad5WT, wild-type Ad5. Labels at the sides of the gel indicate the specific viral proteins; subscripts denote viral types.*

unusual finding that the hybrid heterotypic 100K protein functioned as a wild-type protein in that it produced structurally sound hexons.

Phenotypic characterization. We examined the patterns of synthesis and stabilities of virus-specific polypeptides in cells infected at the nonpermissive temperature to determine what effects the *ts* defect had. It is thought that the affected *ts* polypeptides may not fold properly at the nonpermissive temperature and, therefore, that the affected proteins may be more subject to proteolytic cleavage at 39.5°C (12, 17, 29, 34, 35).

KB cells in a suspension culture were infected with H5*ts*115, H5*ts*116, H5*ts*142 (fiber mutant), H5*ts*147 (hexon mutant), or wild-type Ad5. Mock-infected cells were also examined. At 16 h postinfection, the cells were pulse-labeled for 15 min with [^{35}S]methionine at 39.5°C, and then they were incubated at 39.5°C in a nonradioactive medium containing a 10-fold excess of unlabeled methionine. At different times samples were withdrawn from the cultures and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 2 shows the patterns of synthesis and the stabilities of polypeptides in wild-type virus- and mutant-infected cells. It is clear that both the hexon polypeptides and the 100K polypeptides were unstable at 39.5°C in H5*ts*115- and H5*ts*116-infected cells during the chase period, whereas the remaining viral proteins (e.g., the fiber and penton polypeptides) were not degraded. Indeed, it is striking that the hexon polypeptide chains were degraded to a greater extent than the mutated 100K proteins. Densitometry tracings of the autoradiograms (data not shown) confirmed the visual observations. In contrast, in H5*ts*147-infected cells, in which hexon trimers assembled but accumulated in the cytoplasm, the 100K and hexon polypeptides were both stable at 39.5°C, although the defect of H5*ts*147 is in the hexon gene (15, 21).

Viral multiplication after shift of temperature. To determine the functional effect of the missense mutation on the production of infectious virus, cultures were shifted from 32 to 39.5°C or vice versa at different times after infection. The "shift-up" experiments were used to determine whether a mutant gene product that was synthesized at the permissive temperature could function after a change to the nonpermissive conditions. The "shift-down" experiments were used to determine whether the gene product synthesized at the nonpermissive temperature could renature and function in vivo when the cultures were shifted to the permissive temperature. By adding cycloheximide (10 μg/ml) to one-half of an infected culture, it was also possible to determine whether previously produced proteins could be utilized or whether protein synthesis was necessary for infectious virus to be made after the temperature shift.

Suspension cultures of KB cells were infected with 20 PFU of wild-type virus, H5*ts*115, or H5*ts*116 per cell and incubated at 39.5°C. At 16 h postinfection, the cells were labeled for 15 min with [^{35}S]methionine. The labeled, infected cells were then sedimented, washed, and cultured in unlabeled spinner Eagle minimal essential medium containing 10 times the normal methionine concentration at 39.5°C for 30 min before the shift to 32°C. This chase at the nonpermissive temperature facilitated the depletion of the cellular pool of [^{35}S]methionine available for protein synthesis and allowed the unassembled polypeptide chains to assemble to whatever state possible at 39.5°C. Each culture was then divided into two aliquots, which were placed in fresh spinner flasks and incubated at 32°C; cycloheximide (10 μg/ml) was added to one of these cultures. Samples were withdrawn at dif-

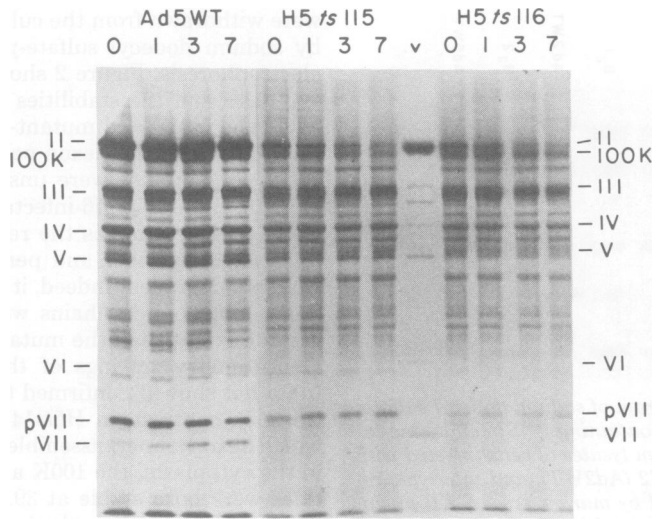

FIG. 2. *Autoradiogram of a sodium dodecyl sulfate-polyacrylamide slab gel showing electrophoretically separated [^{35}S]methionine-labeled polypeptides from extracts of H5ts115-infected, H5ts116-infected, and mock-infected cells pulse-labeled and chased at 39.5°C. KB cells in suspension cultures were infected with 50 PFU of virus per cell at 39.5°C, and then the cultures were incubated for 16 h. The cells were labeled for 15 min with 100 μCi of [^{35}S]methionine per ml and chased for up to 7 h. Samples were withdrawn from the cultures immediately after the pulse-label and at different times during the chase. The cell pellets were washed with pbosphate-buffered saline, disrupted in gel sample buffer by sonication, and boiled for 2 min. The conditions used for electrophoresis are described in the legend to Fig. 1. Lane 0, Sample withdrawn immediately after the pulse; lanes 1, 3, and 7, samples chased for 1, 3, 7 h, respectively; lane v, wild-type Ad5 (Ad5WT) virion.*

ferent times, frozen, thawed six times to release the intracellular virus, and titrated for infectious virus at 32°C by the fluorescent focus assay. In addition, samples were analyzed by sucrose gradient velocity sedimentation to determine whether hexon capsomers were assembled.

Upon shift to the permissive temperature, infectious virus was produced in the absence of cycloheximide but not in its presence, indicating that new protein synthesis was necessary for assembly of infectious virus (Fig. 3). Moreover, hexon polypeptides synthesized at the nonpermissive temperature could be assembled into mature hexons to only a very limited extent upon temperature shift in the presence of cycloheximide (unpublished data).

For temperature shift-up experiments, suspension cultures of KB cells were infected as described above and incubated at 32°C. At 28 h postinfection, which was early in the exponential phase of the viral multiplication cycle at 32°C (6), the infected cultures were divided and placed in a water bath at 39.5°C. Cycloheximide was added to one culture of each type. A small portion of each of the mutant-infected cultures was kept at 32°C as a control. Samples were withdrawn at different times, and the cells were sedimented and resuspended in infecting fluid to remove the cycloheximide before titration at 32°C by the fluorescent focus assay.

In H5*ts*115- and H5*ts*116-infected cells, shift-up to the nonpermissive temperature (Fig. 4) resulted in the arrest of viral production within 1 h; the presence of cycloheximide did not increase the inhibition significantly. The kinetics of inhibition resembled the kinetics of the wild-type virus-infected culture treated with cycloheximide.

Assembly of hexon in vitro. In an attempt to identify the function of the 100K protein, an in vitro protein synthesis system was used to test whether purified wild-type 100K protein added to a 100K protein mutant-infected cell lysate at the nonpermissive temperature allowed hexon assembly to occur. Using a wild-type Ad5-infected cell cytoplasm extract, Wilhelm et al. (41, 42) described the in vitro synthesis of previously initiated polypeptide chains and the assembly of the major multimeric capsid proteins, including the assembly of the hexon polypeptides into trimeric, 12S, immunologically reactive hexons. Although the incorporation of ^{3}H-amino acids into specific adenovirus polypeptides and the assembly of labeled hexon polypeptides into immunoreactive hexons were demonstrated by using wild-type Ad5, when this reaction was carried out with H5*ts*116- or H5*ts*147-infected cell extracts, protein synthesis as measured by isotope incorporation was low, even at 32°C, and newly synthesized immuno-

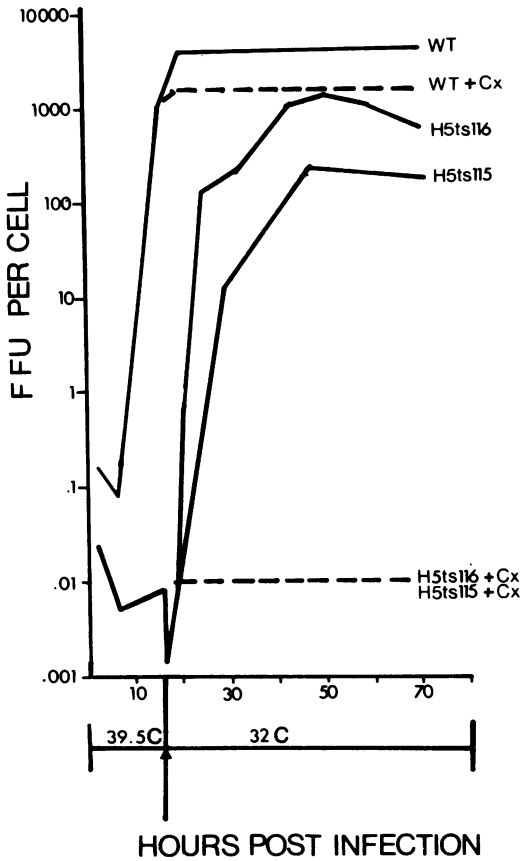

FIG. 3. *Viral multiplication of H5ts115, H5ts116, and wild-type (WT) Ad5 after shift of infected cultures from 39.5 to 32°C. Suspension cultures of KB cells were infected with 30 PFU of virus per cell and incubated at 39.5°C. At 16 h postinfection (indicated by the arrow), the cells were labeled with [^{35}S]methionine for 15 min at 39.5°C as described in the text. After a 30-min chase at 39.5°C, each culture was divided into two aliquots, which were placed in fresh spinner flasks and incubated at 32°C. One H5ts115-infected aliquot, one H5ts116-infected aliquot, and one wild-type Ad5-infected culture aliquot were treated with 10 μg of cycloheximide (Cx) per ml at the time of temperature shift. Samples were withdrawn at different times, frozen and thawed six times, and titrated for infectivity at 32°C by the fluorescent focus assay. FFU, Focus-forming units.*

reactive hexons were not detected (unpublished data). Hence, in vitro complementation could not be accomplished.

Because of the inability to use the reconstituted reaction directly to determine whether the 100K protein was necessary for the assembly of hexons, interference with wild-type hexon assembly by anti-100K immunoglobulin G (IgG) was tested. An IgG fraction purified from anti-100K, anti-fiber, or normal rabbit serum was added to wild-type virus-infected cell cytoplasm, and the mixture was incubated for 30 min on ice, after which the in vitro synthesizing reagents were added and the synthesis reaction was started. After the incorporation of label for 30 min, a *Staphylococcus aureus* suspension was added to the mixture to precipitate the IgG. The supernatant fraction from this precipitation reaction was then mixed with anti-hexon or normal rabbit serum, and the immune complexes

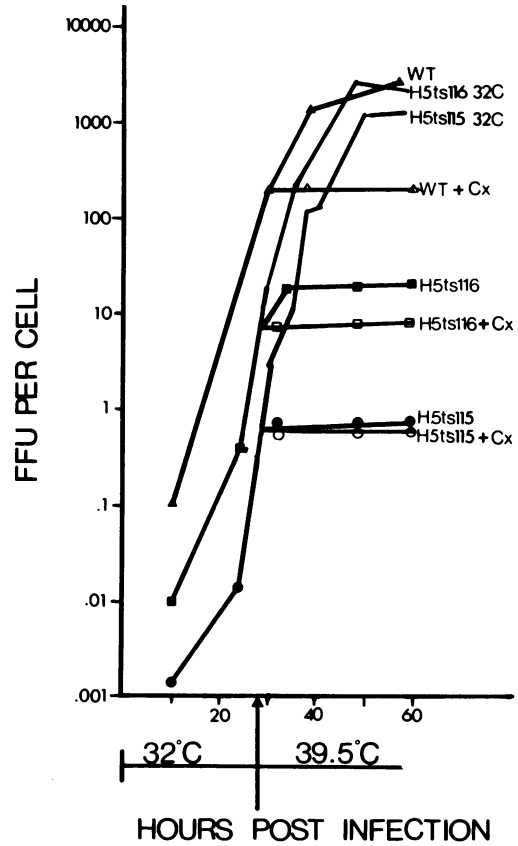

FIG. 4. *Viral multiplication of H5ts115, H5ts116, and wild-type (WT) Ad5 after shift of infected cultures from 32 to 39.5°C. Suspension cultures of KB cells were infected with 20 PFU of virus per cell and incubated at 32°C for 28 h. The cultures were then divided into two aliquots, which were placed in fresh spinner flasks and incubated at 39.5°C. One H5ts115-infected aliquot, one H5ts116-infected aliquot, and one wild-type Ad5-infected aliquot were then treated with 10 μg of cycloheximide (Cx) per ml. Portions of the H5ts115- and H5ts116-infected cell cultures were kept at 32°C (32 C) to determine the viral replication of each at 32°C. At different times, samples were withdrawn, frozen, thawed, and assayed for infectious virus by the fluorescent focus assay. FFU, focus-forming units.*

TABLE 2. *Interference of anti-100K with the assembly of hexon during in vitro synthesis*

Wild-type virus-infected cytoplasm preincubated with:	Total radioactivity (cpm) per 50-μl reaction mixture[a]	Total radioactivity (cpm) precipitated with anti-hexon	Yield of hexon (%)[b]
Anti-100K IgG	64,573	436	32
Anti-100K IgG	61,340	246	19
Anti-100K IgG	56,646	277	23
Anti-100K IgG	79,979	503	30
Anti-fiber IgG	64,348	1,132	85
Normal rabbit IgG	71,698	1,493	100

[a] Acid-precipitable ^{3}H-amino acid counts per minute.

[b] Calculated as follows by assuming that the number of counts per minute immunoprecipitated in the normal rabbit IgG reaction was 100%: theoretical counts per minute of hexon expected = [(1,493) (counts per minute in reaction)]/(71,698); percent yield = [(counts per minute of hexon precipitated)/(theoretical counts per minute of hexon expected)] × 100. Ad5-infected cell lysates were preincubated for 30 min on ice with either anti-100K IgG, anti-fiber IgG, or normal rabbit IgG before the addition of ^{3}H-amino acids, ATP, GTP, creatine phosphate, and creatine phosphokinase. Synthesis proceeded for 30 min at 37°C. After label incorporation, an *S. aureus* suspension was added to the mixture to precipitate the IgG. The supernatant fraction was immunoprecipitated with either normal rabbit antisera or anti-12S hexon serum, and the immune complexes were precipitated by adding *S. aureus*.

were precipitated by adding an *S. aureus* suspension (Table 2). The anti-100K IgG did not interfere significantly with the total amount of ^{3}H-amino acids incorporated, but the amount of immunoreactive hexon detectable was markedly decreased. In contrast, anti-fiber IgG did not affect hexon assembly. These data supported the hypothesis that the 100K protein is necessary for hexon assembly. It should be noted that antibodies to purified native hexons (i.e., 12S trimers) do not immunoprecipitate 100K proteins (25) and that 100K protein antibodies do not immunoprecipitate native 12S hexons (25). In addition, hexon polypeptides derived from purified hexons are not immunoprecipitated by anti-100K antiserum (unpublished data).

DISCUSSION

The data presented characterize two members of a group of conditionally lethal *ts* mutants that cannot form mature hexons at the nonpermissive temperature (i.e., their phenotypes are hexon minus). However, the hexon gene lies between 51.9 and 62.2 map units on the adenovirus genome (5), and, according to the results of marker rescue and genetic recombination analyses, this is not the locus of the gene which produces the H5*ts*115 and H5*ts*116 phenotypes. Physical mapping techniques, such as R-loop (5, 28), cell-free translation (21, 22, 24, 28), and hybrid arrest translation techniques (24, 26), have localized the 100K protein gene between 67 and 75 map units, and the pVIII protein gene between 75 and 79 map units. Since homotypic recombination analyses (11, 16) have indicated that the mutations of H5*ts*115 and H5*ts*116 map within this region, it was necessary to establish unambiguously in which gene the mutations of these alleles resided.

The results of complementation analyses implied that both H5*ts*115 and H5*ts*116 contained mutations in the gene for the 100K protein because both of these mutants failed to complement Ad2$^+$ND1*ts*4, a strain with a mutation previously reported to be in the 100K gene (30). Moreover, homotypic (H5*ts* DNA × wild-type Ad5 restriction fragment DNA) and heterotypic (H5*ts* DNA × Ad2 restriction fragment DNA) marker rescue experiments (unpublished data) clearly indicated that the mutation in H5*ts*116 must be located between coordinates 67 and 70.7 and therefore is not a mutation in the pVIII protein gene; these experiments also showed that the mutation in H5*ts*115 maps between 70 and 75.9 map units and thus cannot a be mutation in the hexon gene. Therefore, since H5*ts*115 and H5*ts*116 are both in the same complementation group, it can be concluded that the mutations in both of these mutants map in the gene encoding the 100K protein. The heterotypic marker rescue accomplished with the Ad2 *Eco*RI-B fragment yielded a *ts*$^+$ recombinant virus which must have resulted from a recombination event in the Ad2 DNA sequences between coordinates 59.5 and 70.7, the region which encodes the N terminus of the 100K protein. Since the heterotypic 100K proteins resulting from this recombination were intermediate in size between the Ad2 and Ad5 100K proteins (Fig. 1), the Ad2 DNA nucleotide sequences recombined must have been at least in part heterologous to the corresponding Ad2 DNA sequences. Whether the actual recombination occurred within homologous or heterologous sequences cannot be determined until the complete nucleotide sequences of Ad2 and Ad5 DNA have been determined in this region of the genome. It is noteworthy that despite the differences in the sizes of the Ad2 and Ad5 100K proteins and thus the differences in the viral genomes encoding these proteins, the heterotypic recombinant protein appeared to function like wild-type 100K protein. Adenovirus heterotypic marker rescue has not been reported previously, and although heterotypic recombination has been described (13, 14, 30), the proteins of

the ts^+ recombinants did not appear to be hybrids (13). Thus, the heterotypic hybrid protein produced by recombination between H5*ts*116 and the Ad2 *Eco*RI fragment B appears to be a unique hybrid protein that is capable of functioning for the production of structurally sound trimeric hexons.

The correlation between the mapping data obtained by the marker rescue technique and the genetic map obtained by recombinational analysis (11) is excellent. Not only does the map distance between the sites of the mutations in strains H5*ts*115 and H5*ts*116 (as determined by marker rescue analysis) correlate with the 1% recombination frequency between these sites, but also the map order of the mutations (as determined by marker rescue and recombination analysis) is the same; i.e., the mutation in strain H5*ts*116 maps to the left of the mutation in strain H5*ts*115.

It is noteworthy that the 100K proteins of H5*ts*115, H5*ts*116, and wild-type Ad5 are phosphorylated similarly at both 32 and 39.5°C (unpublished data), that these proteins do not show any differences in mobility during two-dimensional gel electrophoresis or after partial proteolysis (unpublished data), and that they have the same cellular distribution as determined by indirect immunofluorescence at both temperatures (25). These findings indicate that the presumed single amino acid substitution only subtly affects the overall structure of the protein, but nevertheless prohibits its function.

Degradations of the products of missense mutations have been reported in a number of bacterial and animal virus systems (17, 34, 35). In H5*ts*115- and H5*ts*116-infected cells both the hexon polypeptides and the 100K polypeptides appear to be relatively unstable at the nonpermissive temperature. The hexon polypeptides are rather labile (degraded 75 to 85% in 7 h), yet these polypeptides are not products of a mutated gene. In contrast, the 100K protein, which is the gene product directly affected by the mutation in each allele, is degraded significantly less during 7 h at 39.5°C. It has been suggested that the degradative rates of polypeptides in cells are determined by differences in the conformations of the susceptible proteins (12). The following two observations made with 100K protein mutant-infected cells appear to be consistent with this hypothesis: (i) the 100K protein is subject to protease degradation because its primary structure is altered, which probably affects its folding at the nonpermissive temperature; (ii) the unassembled hexon polypeptides are sensitive to proteolytic cleavage to even a greater extent than the 100K protein, probably because they are not able to fold and assemble into trimers, although the two species of polypeptides do still interact in mutant-infected cells (25). The inability of the hexon polypeptides to assemble may expose more proteolytic cleavage sites in the hexon polypeptide chains than the amino acid substitution in the 100K protein. Thus, in cells infected with the hexon mutants H5*ts*147, H5*ts*135, and H5*ts*128, the partially assembled hexon polypeptides are stable at the nonpermissive temperature (16).

The fact that in H5*ts*115- and H5*ts*116-infected cells infectious virus was not produced and hexon trimers were assembled to only a very limited extent after a shift from the nonpermissive temperature to the permissive temperature in the presence of cycloheximide could have been a consequence of protein degradation of the hexon and 100K polypeptide chains at 39.5°C. These data may also have reflected an inability of the 100K protein made at 39.5°C to assume its functional role after the shift to 32°C, or they may have resulted from defective 100K protein and hexon polypeptide chains associating incorrectly so that after the shift the hexon polypeptides could properly fold and assemble or could dissociate from the 100K protein during assembly. Studies with H5*ts*17 and H5*ts*20 (20), which are now known to be strains with mutations in the 100K protein gene (9), also showed limited hexon assembly after a shift to the permissive temperature in the presence of cycloheximide if time was permitted between the synthesis of the nascent hexon and 100K polypeptide chains at 38.5°C and the shift to 32.5°C. Similar results were obtained with H5*ts*147, a hexon gene mutant (16), whereas H5*ts*142, a fiber gene mutant, could assemble infectious virus in the presence of cycloheximide after the shift from 39.5 to 32°C.

Adenovirus-infected cells also contain a nonstructural 33K polypeptide; Axelrod reported that this polypeptide is a derivative of the 100K protein (3). Since the 33K protein is apparently encoded in the viral DNA at the same location as the carboxy terminus of the 100K protein (24), the missense mutation in the 100K protein gene of H5*ts*115 should also be expressed in the 33K protein, but it should not be expressed in the 33K protein produced in H5*ts*116-infected cells. However, since the phenotypes of H5*ts*115 and H5*ts*116 are almost identical at the nonpermissive temperature, we concluded that either the 33K protein has the same function as the 100K protein or it is not functional. Since almost all of the characterizations of H5*ts*115 and H5*ts*116 have been performed by labeling the hexon and 100K polypeptides with [^{35}S]methionine, the 33K species was not detected.

To obtain definitive evidence that the 100K

protein provides an essential function in the assembly of nascent hexon polypeptide chains into mature hexons, we attempted to develop an in vitro system for the production of hexon in which wild-type 100K protein could complement hexon morphogenesis in 100K protein mutant lysates at the nonpermissive temperature. However, this goal was not accomplished because of poor in vitro synthesis of viral polypeptide chains in the mutant-infected cell extracts (unpublished data). Therefore, to test this hypothesis, in vitro synthesis and assembly of hexons were measured after the 100K protein was precipitated from the wild-type virus-infected cell lysate by specific antibodies. As a control, either antibodies to fiber protein or IgG from normal rabbit serum was used in place of 100K protein antibodies in the preincubation reaction. The quantities of immunoreactive hexons synthesized decreased significantly in the extracts depleted of the 100K protein but not in the controls. These in vitro experiments appeared to strengthen the evidence that the 100K protein and hexon polypeptides interact (25), and the data implied that the reduced production of trimeric hexons resulted from this interaction. Either the 100K protein antibodies removed a large proportion of the hexon polypeptides from the pool and thus reduced hexon production because they co-precipitated 100K and hexon polypeptides from the infected cells extract, or the 100K protein remaining in the reaction mixture was insufficient to effect maximum assembly of hexon polypeptides into mature hexons. The experiments described above do not allow one to distinguish between these two alternatives.

To interpret these experiments further, the characteristics of the antisera used should be emphasized. Antiserum directed against the 100K protein is monospecific; hence, it does not react with purified hexon polypeptide chains or trimeric 12S hexons and does not neutralize virus (25), but in cell lysates it co-immunoprecipitates 100K protein and hexon polypeptides, indicating close association of these polypeptides within cells. Native hexon antiserum (31, 37, 38) reacts only with mature 12S hexons in all reactions, including specific viral neutralization. It should also be noted that hexon polypeptide chain antiserum, which was prepared by immunizing rabbits with denatured, crystallized hexons (31), reacts only with hexon polypeptide chains in purified reaction mixtures and not with purified 100K protein; however, in cell lysates these antibodies precipitate 100K protein and hexon polypeptide chains (unpublished data). Thus, the 100K antiserum could not immunoprecipitate either assembled hexons or unassociated hexon polypeptide chains directly, nor is it likely that the hexon polypeptides did not interact because of steric hindrance or nonspecific trapping since neither the normal rabbit IgG nor the anti-fiber IgG interfered with hexon assembly.

The 100K protein is a viral, nonstructural protein which is synthesized in large amounts during the late phase of a lytic adenovirus infection. The large quantity of 100K protein which is made suggests that it functions stoichiometrically rather than in a catalytic role. Aside from an acetylated N terminus (15), no other modification of the hexon polypeptide is known. Thus, no catalytic role for the 100K protein in hexon morphogenesis is apparent. Moreover, extensive studies could not detect hexon polypeptide modifications, such as cleavage, phosphorylation, and glycosylation (unpublished data). It can be argued that the hexon polypeptides should not require a scaffolding protein to assist in their assembly because they are synthesized in great abundance and their assembly consists of only the association of three identical polypeptide chains. However, it should be recognized that hexon morphogenesis has the following rigid requirements: (i) hexons have both type-specific and family-specific antigenic reactive groups (39, 40), which are only expressed in the trimeric hexons as a result of specific secondary and teritary structural configurations (32); and (ii) the type-specific antigenic reactive site is on the external surface and the family-specific reactive site is on the internal surface when the hexon is assembled into a virion capsid (39). To obtain this rigidly defined conformation, the 100K protein, which has its own unusual structural features, may have to hold the hexon polypeptide chains in a particular conformation which is necessary to permit the specific folding of the chains. As the requirements for hexon assembly are met, the noncovalent bonding properties of the hexon polypeptide are changed so that the 100K protein molecule dissociates from the hexon, similar to the separation of the scaffolding protein of P_{22} bacteriophage (4). It is possible that in cells infected at the nonpermissive temperature with a 100K protein mutant the defective 100K protein synthesized cannot achieve the appropriate folding of the hexon polypeptide chains or the required dissociation from hexon polypeptide chains and, thus, mature hexons cannot assemble.

LITERATURE CITED

1. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. J. Virol. **12**:241-252.
2. **Arrand, J. E.** 1978. Mapping of adenovirus type 5 tem-

perature-sensitive mutations by marker rescue in enhanced double DNA infections. J. Gen. Virol. **41**:573-586.
3. **Axelrod, N.** 1978. Phosphoproteins of adenovirus type 2. Virology **87**:366-383.
4. **Casjens, S., and J. King.** 1975. Virus assembly. Annu. Rev. Biochem. **44**:551-611.
5. **Chow, L. T., J. M. Roberts, J. B. Lewis, and T. R. Broker.** 1977. A map of cytoplasmic RNA transcripts from lytic adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids. Cell **11**:819-836.
6. **Ensinger, M. J., and H. S. Ginsberg.** 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. **10**:328-339.
7. **Everitt, E., L. Lutter, and L. Philipson.** 1975. Structural proteins of adenoviruses. XII. Location and neighbor relationships among proteins of adenovirus type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. Virology **67**:197-208.
8. **Everitt, E., U. Sundquist, U. Petterson, and L. Philipson.** 1973. Structural proteins of adenoviruses. X. Isolation and topography of low-molecular-weight antigens from the virion of adenovirus type 2. Virology **52**:130-147.
9. **Frost, E., and J. Williams.** 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology **91**:39-50.
10. **Ginsberg, H. S., E. Gold, and W. S. Jordan, Jr.** 1955. Tryptose phosphate broth as a supplementary factor for the maintenance of HeLa cell culture. Proc. Soc. Exp. Biol. Med. **89**:66-71.
11. **Ginsberg, H. S., and C. S. H. Young.** 1976. Genetics of adenoviruses, p. 27-86. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol 19. Plenum Press, New York.
12. **Goldberg, A. L., and J. F. Dice.** 1974. Intracellular protein degradation in mammalian and bacterial cells. Annu. Rev. Biochem. **43**:835-869.
13. **Grodzicker, T., C. Anderson, J. Sambrook, and M. B. Mathews.** 1977. The physical locations of structural genes in adenovirus DNA. Virology **80**:111-126.
14. **Grodzicker, T., J. Williams, P. Sharp, and J. Sambrook.** 1974. Physical mapping of temperature-sensitive mutations of adenoviruses. Cold Spring Harbor Symp. Quant. Biol. **39**:439-446.
15. **Jornvall, H., H. Ohlson, and L. Philipson.** 1974. An acetylated N-terminus of adenovirus type 2 hexon protein. Biochem. Biophys. Res. Commun. **56**:304-310.
16. **Kauffman, R. S., and H. S. Ginsberg.** 1976. Characterization of a temperature-sensitive, hexon transport mutant of type 5 adenovirus. J. Virol. **19**:643-658.
17. **Knipe, D., H. F. Lodish, and D. Baltimore.** 1977. Analysis of the defects of temperature-sensitive mutants of vesicular stomatitus virus: intracellular degradation of specific viral proteins. J. Virol. **21**:1140-1148.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
19. **Lawrence, W. C., and H. S. Ginsberg.** 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. **1**:851-867.
20. **Lebowitz, J., and M. S. Horwitz.** 1975. Synthesis and assembly of adenovirus polypeptides. III. Reversible inhibition of hexon assembly in adenovirus type 5 temperature-sensitive mutants. Virology **66**:10-24.
21. **Lewis, J. B., C. W. Anderson, and J. F. Atkins.** 1977. Further mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Cell **12**:37-44.
22. **Lewis, J. B., J. F. Atkins, C. W. Anderson, P. K. Baum, and R. K. Gestland.** 1975. Mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Proc. Natl. Acad. Sci. U.S.A. **72**:1344-1348.
23. **Meyer, J., P. D. Neuwald, S. P. Lai, J. V. Maizel, Jr., and H. Westphal.** 1977. Electron microscopy of late adenovirus type 2 mRNA hybridized to double-stranded viral DNA. J. Virol. **21**:1010-1018.
24. **Miller, J. S., R. P. Ricciardi, B. E. Roberts, B. M. Paterson, and M. B. Matthews.** 1980. Arrangement of messenger RNAs and protein coding sequences in the major late transcription unit of adenovirus 2. J. Mol. Biol. **142**:455-488.
25. **Oosterom-Dragon, E. A., and H. S. Ginsberg.** 1980. Purification and preliminary immunological characterization of the type 5 adenovirus, nonstructural 100,000-dalton protein. J. Virol. **33**:1203-1207.
26. **Paterson, B. M., B. E. Roberts, and E. I. Kuff.** 1977. Structural gene identification and mapping by DNA: mRNA hybrid-arrested cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **74**:4370-4374.
27. **Philipson, L.** 1961. Adenovirus assay by fluorescent cell-counting procedure. Virology **15**:263-268.
28. **Ricciardi, R. P., J. S. Miller, and B. E. Roberts.** 1979. Purification and mapping of specific mRNAs by hybridization-selection and cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **76**:4927-4931.
29. **Russell, W. E., J. J. Skehel, and J. F. Williams.** 1974. Characterization of temperature-sensitive mutants of adenovirus type 5: synthesis of polypeptides in infected cells. J. Gen. Virol. **24**:247-259.
30. **Sambrook, J., J. Williams, P. A. Sharp, and T. Grodzicker.** 1975. Physical mapping of temperature-sensitive mutations of adenoviruses. J. Mol. Biol. **97**:369-390.
31. **Stinski, M. F., and H. S. Ginsberg.** 1974. Antibody to the type 5 adenovirus hexon polypeptide: detection of nascent polypeptides in cytoplasm of infected KB cells. Intervirology **4**:226-236.
32. **Stinski, M. F., and H. S. Ginsberg.** 1975. Hexon polypeptides of type 2, 3, and 5 adenoviruses and their relationship to hexon structure. J. Virol. **15**:898-905.
33. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early mRNAs and proteins on slab gels. J. Mol. Biol. **79**:237-248.
34. **Tegtmeyer, P., J. A. Robb, C. Widmer, and H. L. Ozer.** 1974. Altered protein in metabolism in infection by late *ts*B11 mutants of simian virus 40. J. Virol. **14**: 997-1007.
35. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rudell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. J. Virol. **16**:168-178.
36. **Thiel, J. F., and K. O. Smith.** 1967. Fluorescent focus assay of viruses on cell monolayers in plastic petri plates. Proc. Soc. Exp. Biol. Med. **125**:892-895.
37. **Velicer, L. F., and H. S. Ginsberg.** 1968. Cytoplasmic synthesis of type 5 adenovirus capsid proteins. Proc. Natl. Acad. Sci. U.S.A. **61**:1264-1271.
38. **Velicer, L. F., and H. S. Ginsberg.** 1970. Synthesis, transport, and morphogenesis of type 5 adenovirus capsid proteins. J. Virol. **5**:338-352.
39. **Wilcox, W. C., and H. S. Ginsberg.** 1963. Production of specific neutralizing antibody with soluble antigens of type 5 adenovirus. Proc. Soc. Exp. Biol. Med. **114**:37-42.
40. **Wilcox, W. C., and H. S. Ginsberg.** 1963. Structure of type 5 adenovirus. I. Antigenic relationship of virus structural proteins to virus specific soluble antigens from infected cells. J. Exp. Med. **118**:295-306.
41. **Wilhelm, J. M., and H. S. Ginsberg.** 1972. Synthesis in vitro of type 5 adenovirus capsid proteins. J. Virol. **9**: 973-980.
42. **Wilhelm, J. M., J. J. Jessop, and S. E. Pettitt.** 1978. Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. Bio-

chemistry **17**:1149–1153.

43. **Williams, J., T. Grodzicker, P. Sharp, and J. Sambrook.** 1975. Adenovirus recombination: physical mapping of crossover events. Cell **4**:113–119.
44. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation of HeLa cells by magnesium chloride. J. Gen. Virol. **9**:251–255.
45. **Williams, J. F., M. Gharpure, S. Ustacelebi, and S. MacDonald.** 1971. Isolation of temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. **11**:95–101.
46. **Williams, J. F., C. H. S. Young, and P. E. Austin.** 1974. Genetic analysis of human adenovirus type 5 in permissive and non-permissive cells. Cold Spring Harbor Symp. Quant. Biol. **39**:427–437.