Genetic Analysis of Adenovirus Type 2. Pleiotropic Effects in an Assembly Mutant

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SUMMARY

A new temperature sensitive mutant of adenovirus type 2 was isolated and characterized. H2ts48 is DNA positive but fails to synthesize stable 95K, 80K and Va (Pre-VI) polypeptides. In addition, many polypeptides exhibit reduced stability during pulse-chase experiments. No virion particles of any type are assembled although a new type of intranuclear core-like structure was observed in thin sections by electron microscopy. Hexon polypeptides (120K) are synthesized as demonstrated by SDS-polyacrylamide gel electrophoresis, but immunofluorescence, immunodiffusion and sucrose velocity gradient analyses show that no hexon capsomere antigens (360K) are assembled. Similarly, fibre polypeptides are synthesized normally, but the immunological and sedimentation properties of fibre are abnormal. Because this mutant failed to complement several complementing adenovirus mutants, and depressed the growth of wild type virus, it was concluded that ts48 may be a novel, trans-dominant mutation.

INTRODUCTION

Initial studies with adenoviruses were confined to defining the proteins present in the virion and postulating their function in the architecture of the virus particle (Horwitz, Scharff & Maizel, 1969; Everitt et al. 1973; Everitt & Philipson, 1974; Ishibashi & Maizel, 1974; Rosenwirth et al. 1974; Everitt, Lutter & Philipson, 1975; Nermut, 1975). This led to the isolation of some proteins such as those comprising the hexon, penton base, fibre, and core, and the determination of their chemical and morphological characteristics (Laver, 1970; Anderson, Baum & Gesteland, 1973; Sundquist et al. 1973; Leibowitz & Horwitz, 1974; Dorsett & Ginsberg, 1975; Stinski & Ginsberg, 1975). We have been interested in studying the synthesis of virus coded proteins following productive infection of host cells, and the mechanism by which these proteins are ultimately processed into mature, infectious virions (Liao & Weber, 1969; Weber & Stich, 1969a, b; Weber & Mak, 1970, 1972; Weber, Bégin & Khittoo, 1975; Weber, 1976; Weber, Bégin & Carstens, 1977). Some of these studies have taken advantage of the phenotypic characteristics of conditional lethal temperature sensitive mutants of adenovirus type 2 (Ad2). In this communication we will discuss the isolation and partial characterization of a new temperature sensitive mutant, H2ts48. The results presented here show that although normal DNA synthesis occurs and most late polypeptides are made, no virions are formed at the non-permissive temperature. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of pulse-chase experiments show that specific loss of certain proteins occurs in ts48 infected cells. The complementation data suggest that ts48 is a trans-dominant mutation.

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METHODS

Virus and cells. Human adenovirus type 2 (Ad2) designated as the wild type (WT) was propagated in KB cells and titrated by plaque assay on HEp2 cells, as described previously (Bégin & Weber, 1975; Weber et al. 1975). The procedure for temperature sensitive mutant isolation has also been described (Bégin & Weber, 1975). HEp2 cells were cultured in Dulbecco modified, Eagle's minimal essential medium (DMEM) containing 10% calf serum. Virus infected cells were cultured in DMEM containing 2.5% calf serum and 0.4 mM-arginine.

Virus assembly. HEp2 cells were infected with 1 to 10 p.f.u./cell at 33 °C and adsorbed for 1 h. The inoculum was removed, fresh medium was added and the cells were incubated at 33 °C or 39 °C. The infected cells were harvested by scraping into the medium followed by three cycles of freeze-thawing and low speed centrifugation to remove cell debris. The supernatant was layered on to a pre-formed linear gradient of caesium chloride (1.2 to 1.4 or 1.5 g/ml in 0.05 M-tris-HCl, pH 8.1) and centrifuged at 100000 g for 1.5 h. Fractions were collected dropwise from the bottom of the tube, the density of caesium chloride was determined by refractometry whereas radioactivity was determined as 5% cold trichloroacetic acid (TCA) precipitable counts in toluene based scintillation fluid.

DNA synthesis. Confluent monolayers of HEp2 cells were infected with ts48, WT or mock virus at a multiplicity of 1 to 10 p.f.u./cell. After a 60 min adsorption period at 33 °C, the cells were washed with tris-buffered saline and fresh medium was added. The infected cultures were incubated at 39 °C. After pulsing for 1.5 h with 50 μCi/ml of 3H-thymidine at 18 h post-infection, the cells were washed with tris-buffered saline, scraped off with a rubber policeman, and resuspended in cold tris-buffered saline. Linear 5 to 20% sucrose gradients (in 0.4 M-NaOH, 0.1 M-EDTA, 0.1% sodium lauroyl sarcosine, pH 12.8) were prepared in polyallomer centrifuge tubes essentially as described by Walters & Hildebrand (1975). Two × 10^5 cells were carefully layered on to a hypotonic solution (1% sarcosyl and 100 units/ml heparin) on top of the gradients and were incubated for 15 min at room temperature. Lysing solution (1 M-NaOH, 0.2 M-EDTA) was carefully added, the tubes were left at room temperature for a further 45 min and then centrifuged at 300000 g for 80 min. 14C-formate labelled purified virus was added as a marker of WT DNA. Fractions were collected dropwise from the bottom of the tubes and counted for 5% TCA precipitable radioactivity.

SDS-polyacrylamide gel electrophoresis. Slab gels, 0.75 mm thick, 10.5 cm high, were prepared using a modification of the SDS-disc electrophoresis system described by Maizel (1971). The resolving gel contained 12.5% acrylamide, 0.1% bisacrylamide while the spacer gel contained 5% acrylamide, 1.3% bisacrylamide both in 1.375 M-tris-HCl, pH 8.9. Samples for electrophoresis were dissolved in a stock sample buffer solution (0.05 M-tris-HCl, pH 6.8, 1% SDS, 0.1% mercaptoethanol, 10% glycerol, 0.001% phenol red). Normally, the equivalent of 4000 cells, approx. 20000 ct/min, of 35S-methionine were layered into each well. The gels were run for 1.6 h at constant current of 30 mA, fixed in 40% methanol, 7% acetic acid, dried in vacuo, and autoradiographed with Kodak RP X-omat medical X-ray film.

Electron microscopy. HEp2 cells, infected with 1 to 10 p.f.u./cell of ts48, were incubated for 72 h at 33 °C or 55 h at 39 °C. The cell sheet was washed and then fixed in situ with cold 3.5% glutaraldehyde for 5 min. The cells were then harvested into a pellet, fixed with 1% osmium tetroxide and prepared for examination in the electron microscope as previously described (Carstens & Marusyk, 1975).
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*Immunodiffusion.* The gel consisted of 1.5% noble agar in Beckman B2 buffer. Ten μl samples were added to each well and allowed to diffuse for 18 h at room temperature in a moist atmosphere. The gels were washed for 2 days in 0.15 M-NaCl (2 mg/ml sodium azide), dried under a heat lamp and autoradiographed with Kodak RP X-omat medical X-ray film.

Samples were prepared by pulse labelling infected HEp2 cells for 2 h at 20 h post-infection and 39 °C, and 40 h post-infection at 33 °C. The cells were washed three times with cold tris-buffered saline and then pelleted in a Beckman microfuge. Four × 10⁶ cells were frozen and thawed three times in 0.1 ml of tris-buffered saline, sonicated and then centrifuged to remove the cell debris. The supernatant was used as antigen for immunodiffusion. Antiserum to virions, hexon and fibre antigens were prepared in rabbits from DEAE-cellulose chromatography purified components as described previously (Weber et al. 1975).

*Immunofluorescence.* HEp2 cells, infected with 1 p.f.u./ml of either ts48 or WT for 20 h at 39 °C, were washed in cold phosphate-buffered saline without Ca²⁺ and Mg²⁺ and then fixed in 100% methanol at -70 °C for 18 h. After drying, the cells were covered with rabbit antisera prepared against whole virus, hexon, or fibre antigen (as for immunodiffusion) and incubated at 33 °C. After 30 min, the cells were washed well with PBS (no Ca²⁺ or Mg²⁺) then covered with fluoresceine conjugated goat globulin raised against rabbit globulin and incubated for a further 30 min at 33 °C. The cells were again washed well with PBS, mounted on glass slides with 90% glycerol in PBS and photographed under ultraviolet illumination.

*Virus capsomere assembly.* Linear velocity sucrose gradients as described by Horwitz et al. (1969) were used to investigate capsomere assembly with ts48 at the non-permissive temperature. Infected cells, continuously labelled from 20 h to 48 h at 39 °C or from 36 h to 72 h at 33 °C, were washed with tris-buffered saline, frozen and thawed three times, sonicated briefly and centrifuged at 2000 rev/min to remove cell debris. The clarified supernatant was carefully layered on to linear sucrose gradients of 5 to 25% formed in 12 ml tubes on top of a 1 ml cushion of 56% CsCl, 25% sucrose. Sedimentation was carried out for 14 h at 39000 rev/min (18000 g) in the SB283 rotor (International B60). Fractions were collected dropwise from the bottom of the tube and counted for TCA precipitable radioactivity. Various regions of the gradients were pooled, lyophilized and electrophoresed on SDS-gels.

**RESULTS**

*Isolation of ts48*

Ts48 was isolated by the plaque enlargement technique from a stock of nitrous acid treated wild type (WT) Ad2 (Bégin & Weber, 1975). The mutant was plaque purified twice and grown by passing four times at 33 °C in KB cells. This final stock had a 33/39 °C plaque ratio greater than 10⁶. No normal plaques were seen at 39 °C. At low dilutions the cells showed general cytopathic effects and ‘mini-plaques’ resembling those described previously (Ensinger & Ginsberg, 1972). These mini-plaques remained small even after long incubation. The mutant was also tested for the capacity to form inclusion bodies. Although the proportion of cells containing inclusion bodies was similar at 33 °C and 39 °C, the morphology of the inclusion bodies at 39 °C was abnormal, being very condensed and present in shrunken rather than swollen cells, as compared with WT inclusions.
Fig. 1. Caesium chloride velocity-equilibrium gradient analysis of ts48 grown at 33 °C (■) and 39 °C (●). (a) Ts48 infected cells labelled with 25 μCi/ml of 35S-methionine from 12 h to 20 h p.i. at 39 °C, or from 22 h to 40 h p.i. at 33 °C, were chased for 40 h or 30 h at 39 °C and 33 °C respectively, in the presence of unlabelled methionine before ultracentrifugation. (b) and (c) Ts48 infected cells were pulse labelled with 100 μCi/ml of 3H-thymidine for 1.5 h at 12 h p.i. at 39 °C or for 2 h at 30 h p.i. at 33 °C. The media was then removed and the cells were continuously labelled with fresh media containing 25 μCi/ml 35S-methionine until harvested at 40 h or 30 h later at 39 °C and 33 °C respectively. (b) Shows the pattern of 35S-methionine while (c) shows the pattern of 3H-thymidine after correcting for spillover from 35S. •—•, Density.
Fig. 2. Alkaline sucrose gradient analysis of DNA synthesized in ts48, WT, and mock infected cells. Ts48, WT or mock infected cells, grown at 39 °C were pulse labelled with 100 μCi/ml of 3H-thymidine for 1.5 h at 18 h p.i. Following the pulse, the cell sheets were washed with tris-buffered saline before samples of 2 x 10⁶ cells were prepared and carefully layered on to pre-formed sucrose gradients (5 to 20 % sucrose in 0.4 M-NaOH, 0.1 M-EDTA, 0.1 % sarcosyl). Purified ³¹C-formate labelled virus was added as a source of marker DNA. The arrow indicates the position of the 34S marker DNA peak. Ts48 (□); WT (■); mock (▲).

Absence of particle formation

To determine whether or not stable virus particles were made at 39 °C, two types of experiments were carried out. In the first, ts48 infected cells were labelled from 12 to 20 h post infection (p.i.) with 25 μCi/ml of ³⁵S-methionine and chased for 40 h in the presence of 100 × concentration of unlabelled methionine containing medium. These cells were frozen and thawed three to four times, the cell debris was removed by low speed centrifugation, and the supernatant was layered on top of a pre-formed linear gradient of CsCl. After centrifugation at 100,000 g for 1.5 h, fractions were collected dropwise from the bottom of the tube and counted for cold TCA precipitable radioactivity. As shown in Fig. 1(a), at the non-permissive temperature a single stable peak of radioactivity sediments to a density of 1.28 g/ml, corresponding to top components while no mature virions were detected in the 1.34 g/ml portion of the gradient. The number of counts in the soluble component region was reduced, suggesting that either there was little protein synthesis occurring, or polypeptides were being synthesized normally but that they were unstable and were degraded.

A second type of experiment was done to determine whether there were some intermediate but unstable capsid complexes present and also to determine whether there was any virus DNA associated with these complexes. Ts48 infected cells were pulse labelled...
Fig. 3. SDS-polyacrylamide slab gel autoradiogram of ts48, WT and mock infected HEp2 cells 35S-methionine labelled for \(1\) h at \(39\) °C or \(1.5\) h at \(33\) °C (P) or chased in the presence of non-radioactive medium (C). In samples shifted from \(39\) °C to \(33\) °C or from \(33\) °C to \(39\) °C, the number indicates the hours after shifting before the samples were harvested. The cells were labelled at \(20\) h and \(40\) h p.i., at \(39\) °C and \(33\) °C, respectively.

With \(^3\)H-thymidine (100 \(\mu\)Ci/ml) for \(1.5\) h at \(12\) h p.i. at \(39\) °C or \(2\) h at \(30\) h p.i. at \(33\) °C. The medium was then removed and the cells were grown in the presence of \(^35\)S-methionine (25 \(\mu\)Ci/ml) for a further \(40\) h at \(39\) °C or \(30\) h at \(33\) °C.

A large soluble component pool was obtained (Fig. 1b) which tended to smear into the \(1.28\) g/ml peak. Again, no virion peak could be detected. These results suggested that the polypeptides were being synthesized, but that they were somewhat unstable at \(39\) °C.

When the \(^3\)H-thymidine counts were plotted, after correcting for \(^35\)S spillover, no counts were seen associated with the \(1.28\) g/ml peak at \(39\) °C (Fig. 1c), indicating that relatively little virus DNA was present in this complex.

Whether the protein complex we observed does in fact consist of top components, was determined by electron microscopy of negatively stained preparations from the peaks in CsCl gradients. Neither empty shells nor any other kind of organized structure could be detected at the non-permissive temperature (results not shown).
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Since virus particles were evidently not assembled in ts48 infected cells, it became necessary to determine whether virus DNA was indeed synthesized.

Ts48 infected cells were pulse labelled for 1·5 h with 10 µCi/ml of ³H-thymidine at 12 h p.i. Samples of cells were then removed and carefully layered on top of pre-formed alkaline sucrose gradients. After lysing at room temperature for 1 h, the gradients were centrifuged at 300,000 g for 80 min, fractions were collected dropwise from the bottom of the tube and counted for cold TCA precipitable radioactivity. As shown in Fig. 2, 34S DNA was apparently present in ts48, indicating that DNA synthesis was normal at 39 °C. Consequently, the failure of virion assembly is not due to a lack of DNA synthesis, but some other factors in later stages of the infection process.
Virus-specific polypeptide synthesis

As ts48 was shown to replicate DNA but failed to assemble virions at 39 °C, it was necessary to determine whether late proteins were synthesized normally at the non-permissive temperature. Ts48, WT, and mock infected cells were pulse labelled with 25 μCi/ml of 35S-methionine (1 h at 20 h p.i. at 39 °C or 1.5 h at 40 h post-infection at 33 °C). Following the labelling period, the cells were either harvested immediately by dissolving in sample buffer, or chased in medium containing 100 x concentration of unlabelled methionine for a further 24 h. The pulsed and chased samples were analysed by SDS gel electrophoresis and the radioactive band pattern was revealed by autoradiography.

Fig. 3 shows a typical gel pattern of ts48 infected cell extracts along with identically treated WT and mock infected cells. The autoradiogram was scanned at 550 nm and the densitometer tracing profiles are shown in Fig. 4. As is evident by comparing the tracing of ts48 pulsed at 33 °C to that pulsed at 39 °C, there was an overall reduction in labelled virus proteins at 39 °C, indicated by the reduced area under the peaks of most polypeptides. This apparently non-specific reduction in virus polypeptide synthesis was observed in many analyses. The extent of reduction in protein synthesis varied from experiment to experiment, increasing with multiplicity and the passage level of ts48. To minimize this variation, the experiments reported here were conducted with virus at the second or third passage levels. Except for this generalized reduction in radioactive incorporation, all the polypeptides labelled in the pulse at 33 °C were also present in the pulse at 39 °C and both patterns were similar to the WT infected cell extracts pulsed at 33 or 39 °C. Thus, ts48 seems to synthesize all late polypeptides which can be detected by SDS gel electrophoresis, although their synthesis is either reduced or the polypeptides are unstable. The possibility of non-specific loss of proteins from infected cells is excluded because both the medium and cells were collected and dissolved in SDS sample solution in some experiments.

As we have shown before (Weber, 1976), some processing events, associated with normal virus maturation, do not occur with temperature sensitive mutants which do not make mature particles. Ts48 infected cells, chased for 24 h at 39 °C (Fig. 3) do not cleave certain polypeptides: PreVII, the precursor of the major core protein VII, is not cleaved; Va, the presumptive precursor of polypeptide VI, is very weakly labelled at 39 °C, and totally disappears in the chase, yet no VI appears; Vb is also not processed. Polypeptides VII, and X-XII, normally present after a long chase, are all absent. Several processing events do occur in ts48 chased at the non-permissive temperature. The mobility of 72K, the DNA binding protein, is slightly reduced, 55K appears and 11K disappears as in the WT following the chase. Polypeptides 95K and 80K are reduced or absent in ts48 at 39 °C. The function of these latter two proteins is unknown but they are apparently not necessary for virus infectivity as they are also absent at 33 °C where infectious virus is produced.

It was of interest to determine whether the temperature sensitive defect could be corrected by shifting the infected cells grown at the non-permissive temperature to the permissive temperature. After 10 h, 12 h, 15 h, 18 h and 20 h at 39 °C, ts48, WT and mock infected cells were pulsed for 1 h with 35S-methionine. They were then shifted to 33 °C and chased for either 24 h or 48 h in the presence of fresh medium containing 100 x concentration of unlabelled methionine. Similarly, cells infected at 33 °C were pulsed for 1.5 h (24 h, 30 h and 40 h p.i.) then shifted to 39 °C and chased for 4 h or 24 h. The chased cells were then harvested in sample buffer and analysed by gel electrophoresis. The band pattern was revealed by autoradiography and is shown in Fig. 3.

Cells infected at 39 °C and subsequently shifted to 33 °C gave identical gel patterns
Fig. 5. Caesium chloride velocity-equilibrium gradients of ts48. (a) Ts48 infected cells, grown at 39 °C, labelled with 35S-methionine for 1 h at 20 h p.i. and chased in the presence of excess unlabelled methionine for 4 h at 39 °C. (b) Ts48 infected cells grown at 39 °C, pulse labelled with 35S-methionine for 1 h at 20 h p.i. then shifted to 33 °C and chased for 8 h in the presence of excess unlabelled methionine. (c) Ts48 infected cells, grown at 39 °C, shifted to 33 °C at 20 h p.i., labelled with 35S-methionine for 2 h and chased for a further 20 h at 33 °C in the presence of excess unlabelled methionine. (d) Ts48 infected cells grown at 33 °C, labelled for 2 h at 40 h p.i. and chased for 8 h in the presence of excess unlabelled methionine. The arrow marks the position of marker WT virions.

after 24 h or 48 h chases, both of which were different from WT infected cells. Va was very reduced, there was no detectable VI, VII, VIII, or X–XII while 55K seemed to be present at an increased level when compared to Wt infected cells. Furthermore, as is frequently the case, polypeptides 95K and 80K are reduced as compared with WT. Thus, these patterns were identical to ts48 infected cells grown continuously at 39 °C, indicating that the temperature effect could not be overcome by shifting to the permissive temperature late after infection.

The cells infected at 33 °C, and then chased at 39 °C for either 4 h or 24 h gave slightly different gel patterns from those shifted down. As would be expected, these samples were similar to samples simply pulsed at 33 °C, that is, all polypeptides were labelled. A small amount of processing had occurred after 4 h, but this did not change after 24 h. Specifically, traces of VII, VIII, and X–XII can be seen in these samples. Presumably, this processing was already initiated at 33 °C and occurred before the temperature sensitive effect was exerted after the shift to 39 °C. Va does become reduced after the 24 h chase, but there is no concomitant appearance of polypeptide VI. Eleven K was also reduced after the 24 h chase but did not completely disappear as was the case with WT infected cells. These results indicate that the temperature sensitive protein is synthesized throughout the infection cycle.
at 39 °C, it is not resucuable by shifting down and becomes inactivated very quickly upon shifting from 33 to 39 °C. The SDS-PAGE analysis indicated that no processing of labelled polypeptides had occurred after shift down but it was not clear whether or not some of the radioactive proteins were assembled into mature virions. To investigate this possibility, caesium chloride velocity-equilibrium gradients were performed on ts48 infected cell extracts following a variety of labelling procedures (Fig. 5). Ts48 infected cells, pulse labelled for 1 h at 20 h p.i. at 39 °C and chased for 4 h at 39 °C (Fig. 5a) or pulse labelled for 2 h at 40 h p.i. at 33 °C and chased for 8 h at 33 °C (Fig. 5d) were used as controls.

Ts48 infected cells, pulse labelled for 1 h at 20 h p.i. at 39 °C, immediately shifted to 33 °C and chased for 8 h or 22 h (Fig. 5b) in the presence of excess unlabelled methionine, revealed no incorporation of ^35S-methionine into mature virions. However, when ts48 infected cells, grown at 39 °C for 20 h, were shifted to 33 °C, labelled for 2 h and chased for 20 h at 33 °C (Fig. 5c) some radioactive labelled virions could be detected. This would indicate that only proteins synthesized at 33 °C were capable of being synthesized into virions. No rescue of proteins synthesized at 39 °C was observed by shifting down, implying that the temperature sensitive step in assembly was not reversible late after infection.
Subcellular distribution of virus-induced polypeptides

It has been shown that adenovirus proteins are synthesized in the cytoplasm of infected cells and are subsequently transported to the nucleus where assembly of virions occurs (Velicer & Ginsberg, 1968). To study the distribution of virus-induced proteins, cells infected with ts48 were pulse labelled for 1 h at 20 h p.i. with 25 μCi/ml of 35S-methionine and either harvested immediately (pulse) or chased in the presence of 100× concentration of unlabelled methionine as discussed in the previous section. The cytoplasm and nuclei were separated as follows: the cells were rinsed with distilled water, then scraped off in 0.02 M-tris-HCl, pH 7.4, 0.5% Triton X-100, 0.02 M-EDTA, agitated on a vortex mixer for 2 min and then centrifuged in a Beckman microfuge. The supernatant containing the cytoplasmic fraction was removed and air dried at room temperature overnight before SDS sample buffer was added, while the pelleted nuclei were immediately dissolved in the SDS sample buffer after extraction. The samples were then analysed by gel electrophoresis and the radioactive band pattern was revealed by autoradiography.

The gel pattern of ts48 infected nuclei (Fig. 6) pulsed at 33 °C was very similar to the pattern obtained with WT although polypeptides 95K and 80K were virtually absent in the ts48 infected nuclei. Polypeptides 80K, 72K, 55K and 36K were found only in the nuclear fraction with both ts48 and WT. No chase was done at 33 °C in these particular experiments. At 39 °C, the pulse with ts48 revealed an absence of 95K, 80K and a reduced amount of Va. After a long (24 h) chase, Va completely disappeared while VI, VII, VIII and X–XII were all absent. These results support those obtained with whole cells described above.

The cytoplasmic band patterns of ts48 following the pulse at 33 °C were identical to the WT cytoplasmic fraction but at the same time, both of these gave an unexpected result. Polypeptide PVII was absent while polypeptide VII was present as were small quantities of VI, VIII, and X–XII. This result was never observed in whole cell extracts harvested immediately after the pulse and may thus indicate some in vitro processing in the cytoplasmic fraction while it was desiccated at room temperature before the SDS sample buffer was added.

After the samples were pulse labelled at 39 °C, a reduction in some polypeptides including II, Va, and Vb was observed. Following a long chase, there was an even greater reduction in all polypeptides except III and IV. The cellular proteins remain labelled at approximately the same level. This was in marked contrast to the long chase cytoplasmic extracts of WT infected cells where, processing aside, constant levels of these same polypeptides were seen. As some of these proteins are present only in virions, the WT-cytoplasm chase gel pattern may be contaminated by whole virus which has leaked out of the nucleus during the long chase and during extraction. As shown by the results of CsCl equilibrium gradients, no virions are produced at 39 °C with ts48, and as can be seen here, there is no evidence for their presence in cytoplasmic extracts.

Electron microscopy

HEp2 cells infected with ts48 at either 33 or 39 °C were harvested at late times after infection and prepared for examination in the electron microscope. As can be seen in Fig. 7, there was a striking morphological difference between the two samples. At 33 °C (Fig. 7a), ts48 infected cells revealed the ‘normal’ appearance of an adenovirus infected cell: a large, swollen nucleus, many virus particles, and various virus-induced inclusion bodies (Weber & Stich, 1969a). At 39 °C (Fig. 7b), no virus particles were observed, confirming
Fig. 7. (a) Electron micrograph of HEp2 cells, infected with ts48 for 72 h at 33 °C. This morphology is indistinguishable from that of cells infected with WT at 33 °C or 39 °C. (b) Electron micrograph of HEp2 cell, infected with ts48 for 55 h at 39 °C. Note electron-dense masses of material and extensive vacuolization in the cytoplasm, and angular appearance of nucleus filled with electron-dense, core-like particles in lieu of virus particles. (c) High magnification electron micrograph of nucleus, with dense, amorphous structures.

the results obtained by CsCl gradients and analysis on SDS slab gels. Instead, roughly spherical, amorphous electron-dense structures, slightly larger than whole virions, were observed, possibly consisting of virus DNA and some proteins (Fig. 7c). Although such structures are not seen in WT infected cells, they could represent a short-lived intermediate in WT virus maturation.

The cytoplasm of ts48 infected cells at 39 °C contained extensive areas of uniformly staining electron-dense material, not normally seen in adenovirus infected cells nor with ts48 infected cells at 33 °C. The nature or origin of this material is unknown, although it is most likely virus induced as no stimulation of cellular proteins was seen on SDS gels of ts48 infected cells. Extensive vacuolation of the cytoplasm and a contracted appearance of the nucleus are characteristic aspects of ts48 infected cells. Similar morphological aspects were also noted in ts3 infected cells (Weber et al. 1977).

**Autoradiography**

As virus proteins are synthesized in the cytoplasm and transported to the nucleus, it should be possible to pulse label infected cells with a radioactive protein marker and then observe its migration into the nucleus (Velicer & Ginsberg, 1968). HEp2 cells were infected with ts48 at 39 °C. At 20 h p.i. the cells were pulsed for 30 s or 2 min with 50 µCi/ml of
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Fig. 8. Autoradiograms of gel immunodiffusion patterns. Ts48, ts3, and WT 35S-methionine labelled whole cell extracts were tested with antisera prepared against whole Ad2 virions, Ad2 hexon capsomeres and Ad2 fibre capsomere. 1, Ts48 39 °C; 2, ts48 33 °C; 3, ts3 39 °C; 4, WT 33 °C; 5, ts3 33 °C; 6, WT 39 °C. (a) Central well contained anti-Ad2-fibre serum, (b) central well contained anti-Ad2-virion serum, (c) central well contained anti-Ad2-hexon serum.

3H-mixed amino acids. After various periods of cold chase, the cells were washed three times with cold tris-buffered saline, fixed for 10 min with Carnoy’s fixative, mounted on glass slides, and covered with autoradiographic film. After 1 week exposure, the cells were examined microscopically to determine if the label had been transported to the nucleus. The results (not shown) indicate that protein transport was only negligibly reduced in ts48. Alternatively, possibly most proteins are normally transported while one or two polypeptide species are not transported.

Immunodiffusion

Fig. 8 shows the immunodiffusion patterns obtained with ts48 and WT antigens prepared at 33 and 39 °C, and antiserum to virions, hexon and fibre antigens. Clearly, while fibre is reduced when reacted against either fibre or virion antiserum, the hexon precipitin line is totally absent in both these reactions in ts48 at 39 °C. Since the SDS-PAGE analysis demonstrated the synthesis of hexon polypeptide, we may conclude from these results that ts48 fails to trimerize these monomers into stable hexon antigens.

Immunofluorescence

The indirect immunofluorescent staining with the virion antiserum (Fig. 9a and d), the fibre antiserum (Fig. 9b and e) and the hexon capsid antiserum (Fig. 9c and f) were all reduced with ts48 infected cells, grown at 39 °C. There were some very faint perinuclear areas visible in the ts48 infected cells stained with the virion and hexon antiserum, but this fluorescence was negligible compared to the very strong nuclear fluorescence observed with the WT infected cells. These results confirm the immunodiffusion data that ts48 is defective for the synthesis of normal amounts of immunologically reactive fibre antigen and completely defective for the synthesis of antigenic hexon proteins.

Assembly of capsomeres

The results of the immunodiffusion and immunofluorescence experiments described indicated that no immunologically active hexon and very little fibre was present in ts48 infected cells at 39 °C. To confirm these data, velocity sedimentation of cell extracts through
Fig. 9. Immunofluorescence photomicrographs of ts48 and WT infected HEp2 cells at 39°C. Cells, fixed at 20 h p.i., were treated with anti-hexon, anti-fibre or anti-virion serum and subsequently stained with fluorescein conjugated goat globulin raised against rabbit globulin. (a) and (d) WT and ts48 infected cells respectively, treated with anti-Ad2-virus serum; (b) and (e) WT and ts48 infected cells respectively, treated with anti-Ad2-fibre serum; (c) and (f) WT and ts48 infected cells respectively, treated with anti-Ad2-hexon serum.
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Fig. 10. Autoradiogram after electrophoresis of cell pellets. WT and ts48 infected cells labelled for 24 h at 39 °C with 35S-methionine were harvested, sonicated for 2 min and the cell debris was treated with 0·5 % sodium deoxycholate (NaDoc), 0·5 % sodium dodecyl sulphate (SDS) or 1 % Triton X-100 (Triton). The cell debris was collected by centrifugation at 2000 rev/min and prepared for electrophoresis. Ts48 infected cell extract pulsed for 1 h and chased for 24 h at 33 °C was included as a marker of virus polypeptides (C).
Fig. 11. Assembly of virus capsomeres at 39 °C in ts48 versus WT infected cells. HEp2 cells, infected with either ts48 or WT, were labelled with 35S-methionine from 20 h to 48 h p.i. The infected cells were then washed, sonicated for 2 min and the debris was pelleted by centrifugation at 2000 rev/min for 10 min. The supernatant was saved and the pellets were mixed with either 0.5% sodium deoxycholate (a) or 0.5% sodium dodecyl sulphate (b) for 1 min on a vortex mixer. The debris was again removed by centrifugation at 2000 rev/min and the resulting supernatants were pooled with the sonication supernatants and analysed on sucrose gradients as described in Methods. ■—■, Ts48; □—□, WT.

Sucrose gradients was carried out. At 20 h p.i. at 39 °C, WT or ts48 infected HEp2 cells were labelled for 24 h with 35S-methionine. The cells were washed, sonicated for 2 min, the cell debris was removed by centrifugation at 2000 rev/min, and the clarified supernatant was sedimented through 5 to 25% linear sucrose gradients. These gradients revealed that little assembly of hexon, penton or fibre subunits was occurring in ts48 infected cells at 39 °C (results not shown). However, when the cell debris was examined by SDS–PAGE, rather large amounts of hexon, penton base and fibre polypeptides were still present (Fig. 10), especially in ts48 infected cells. To ensure that the lack of capsomeres on sucrose gradients was not due to binding or trapping of these components in ts48 infected cells, the cell debris was treated with various detergents including 0.5% NaDoc, 0.5% SDS and 1% Triton X-100. The supernatants of these various extraction procedures were added to the supernatants obtained after sonication as described above, and were again analysed on sucrose gradients. As can be seen in Fig. 11, showing the results obtained with NaDoc and SDS, no assembled capsomeres were seen with ts48 infected cells although there was an increase in radioactivity in the top portion of the gradient of cells treated with SDS similar to that seen in the WT infected cells. The cell debris was well solubilized by SDS,
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<table>
<thead>
<tr>
<th>WT</th>
<th>NaDoc</th>
<th>SDS</th>
<th>WT</th>
<th>NaDoc</th>
<th>SDS</th>
<th>TS1</th>
<th>NaDoc</th>
<th>SDS</th>
<th>TS1</th>
<th>NaDoc</th>
<th>SDS</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 12. SDS-polyacrylamide slab gel autoradiogram of pooled fractions of sucrose gradients shown in Fig. 11. The pooled fractions, corresponding to numbers 1 to 7 were precipitated with 10% TCA, washed with acetone and analysed by SDS slab gel electrophoresis. NaDoc, cells treated with 0.5% sodium deoxycholate; SDS, cells treated with 0.5% sodium dodecyl sulphate; TS1, ts48 infected cells labelled for 8 h with 35S-methionine, chased for 24 h in the presence of excess unlabelled methionine; TS2, ts48 infected cells, continuously labelled with 35S-methionine for 32 h.

As shown by the autoradiogram in Fig. 10. Only small amounts of the virus polypeptides were present when compared with cells treated with either NaDoc or Triton (Fig. 10).

To correctly identify the peaks obtained in the sucrose gradients, various fractions were pooled and analysed by SDS–PAGE. As can be seen in Fig. 12, in WT infected cells treated with either NaDoc or SDS, pool 1 seemed to consist of polypeptides found in virions, pool 2 consisted mainly of hexon, pool 3 consisted of penton (penton base and fibre), pools 4 and 5 contained mainly fibre, while pools 6 and 7 consisted principally of polypeptide IX.

The ts48 generated polypeptide pattern is quantitatively quite different. There is an almost complete absence of hexon and penton, while pool 4, which normally consists of fibre, did not contain enough radioactivity to warrant electrophoresis. Surprisingly pool 1 of ts48 contained large amounts of fibre. Since the polypeptide pattern of pool 1 is unlike that of virions, the fibre present in this fast-sedimenting and detergent-resistant component represents an anomalous behaviour of fibre hitherto unobserved. Evidently, soluble 6S fibre and fibre complexed with penton base are completely missing. The absence of penton is probably a consequence of the absence of 6S fibre, since penton base polypeptides are observed in the slow sedimenting pools 5 and 6 (Fig. 12).

Cells treated with SDS revealed a shift of some proteins (V, VI, PVII, VII) from the fast sedimenting peak (pool 1) to the top of the gradient (pools 6 and 7) indicating that these
Table 1. Intratypic and intertypic complementation with H2ts48

<table>
<thead>
<tr>
<th>Virus infection</th>
<th>Virus yield (p.f.u./ml)</th>
<th>Complementation index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33 °C</td>
<td>39 °C</td>
</tr>
<tr>
<td>H2ts1</td>
<td>2.3 x 10^8</td>
<td>6.6 ± 10^8</td>
</tr>
<tr>
<td>H2ts1 + H2ts48</td>
<td>—</td>
<td>6.0 x 10^7</td>
</tr>
<tr>
<td>H2ts3</td>
<td>1.0 x 10^7</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>H2ts3 + H2ts48</td>
<td>—</td>
<td>8.0 x 10^5</td>
</tr>
<tr>
<td>H2ts4</td>
<td>9.0 x 10^7</td>
<td>1.6 x 10^6</td>
</tr>
<tr>
<td>H2ts4 + H2ts48</td>
<td>—</td>
<td>3.0 x 10^7</td>
</tr>
<tr>
<td>H5ts1</td>
<td>2.7 x 10^8</td>
<td>1.0 x 10^7</td>
</tr>
<tr>
<td>H5ts1 + H2ts48</td>
<td>—</td>
<td>2.7 x 10^7</td>
</tr>
<tr>
<td>H5ts2</td>
<td>1.1 x 10^8</td>
<td>7.0 x 10^7</td>
</tr>
<tr>
<td>H5ts2 + H2ts48</td>
<td>—</td>
<td>2.0 x 10^6</td>
</tr>
<tr>
<td>H5ts22</td>
<td>2.5 x 10^8</td>
<td>7.5 x 10^6</td>
</tr>
<tr>
<td>H5ts22 + H2ts48</td>
<td>—</td>
<td>2.7 x 10^6</td>
</tr>
<tr>
<td>H2ts1 + H2ts4</td>
<td>—</td>
<td>9.0 x 10^5</td>
</tr>
<tr>
<td>H2ts48</td>
<td>8.4 x 10^8</td>
<td>9.0 x 10^5</td>
</tr>
<tr>
<td>Ad2</td>
<td>1.0 x 10^8</td>
<td>1.5 x 10^6</td>
</tr>
<tr>
<td>Ad2 + H2ts48</td>
<td>5.0 x 10^8</td>
<td>6.0 x 10^5</td>
</tr>
</tbody>
</table>

* The procedures used to test for complementation have been described previously (Bégin & Weber, 1975). Briefly, HEp2 cells were infected either singly with a multiplicity of infection of 20 p.f.u./cell or doubly with 10 p.f.u./cell of each virus. Single infections were incubated at 33 and 39 °C, while mixed infections were incubated at 39 °C (except the mixed infection with Ad2 which was incubated at both temperatures). At the end of 2 days (39 °C) or 3 days (33 °C) virus was harvested and titrated by plaque formation on HEp2 cells at 33 °C. The complementation index was calculated by dividing the mixed infection yield with the sum of the two corresponding single infection yields.

proteins from the virions, empty virions and possible fast sedimenting subviral complexes had been extracted and solubilized.

The results of ts48 treated with NaDoc indicate a small difference between samples which were labelled for 8 h (TS1) and those which were continuously labelled (TS3). A small amount of hexon polypeptide was present in the continuously labelled sample (TS3) indicating that some hexon was assembled. However, it would appear that this hexon was unstable as no hexon polypeptide was seen in the sample which was labelled and then chased (TS1).

In summary, these results show that ts48 fails to assemble the 12S hexon capsomere. In addition, they also demonstrate an abnormal sedimentation behaviour of the fibre which is not seen in WT infected cells.

Complementation

In order to investigate the genetic nature of the ts48 mutation, especially its relationship to selected Ad2 and Ad5 ts mutants, we performed complementation experiments as described previously (Bégin & Weber, 1975). Under conditions where good complementation was observed between mutants known to complement (H2ts1 × H2ts3; H2ts3 × H2ts4), we repeatedly failed to observe complementation of any of these mutants with ts48 (Table 1). Although the results were variable between different experiments, it appeared that weak complementation may occur with H2ts1 and H2ts4. Significantly, ts48 failed to complement the three well characterized and well separated Ad5 ts mutants, H5ts1, H5ts2 and H5ts22, which are in three separate complementation groups (Russell, Newman & Williams, 1972). Because of this lack of complementation, the question arose whether ts48 is a trans-acting, dominant mutation. To test this hypothesis we titrated the yields from co-infections
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of ts48 with Ad2 wild type virus. Surprisingly, the mixed infection yield was 250-fold less than expected at 39 °C, while it was normal at 33 °C (Table 1). Therefore it appears conceivable that ts48 fails to complement properly due to this dominant interfering effect.

DISCUSSION

We have studied the phenotype of a temperature sensitive mutant, H2ts48, at 33 and 39 °C. At 39 °C, ts48 did not assemble precursor or mature virus particles as determined by caesium chloride equilibrium gradients and electron microscopy. However, electron microscopic examination of thin sections of cells infected with ts48 at 39 °C revealed electron-dense, core-like structures (Brown et al. 1975). Studies are in progress to isolate these structures from infected cells and to characterize their chemical composition. Such structures are not observed in WT infected cells, hence they may represent the accumulation of a normally short lived intermediate. Similar structures have also been observed with ts3 (Weber et al. 1977), which, however, exhibits a distinct polypeptide pattern from ts48.

Alkaline sucrose gradients (Fig. 2) revealed that normal virus DNA synthesis occurs with ts48 at 39 °C, indicating that the temperature sensitive mutation is involved in a late function of virus multiplication. This is supported by the data obtained from SDS gel electrophoresis of pulse labelled infected cell extracts. With the exception of the products of the maturation cleavages (Weber, 1976), all late polypeptides except 95K and 80K were present at 39 °C. As 95K and 80K are also reduced or absent at 33 °C in ts48, the role of these proteins remains obscure.

The results of pulse-chase experiments lend support to the hypothesis that cleavage of certain proteins, specifically Va and PVII, is a late step in virus assembly and occurs only in the presence of encapsidated DNA (Ishibashi & Maizel, 1974; Weber, 1976). Apparently spontaneous in vitro cleavage of PVII to VII occurs readily, but independently of the Va to VI cleavage event, which was not observed to take place (Fig. 6, track 9). The independence of these cleavage events were noted previously in vivo with ts3, where Va and Vb were processed but PVII was not (Weber et al. 1977).

Polypeptide Va appeared to be strongly affected by the temperature sensitive mutation. Very little change in the level of this protein occurred at 33 °C, even after a long chase, whereas at 39 °C, Va was weakly labelled and almost totally disappeared after a long chase, without concomitant appearance of VI. We may conclude that Va was degraded at the non-permissive temperature. Although VI could be seen in only small quantities in infected cell extracts after a long chase at 33 °C, it was present in sufficient quantities for virus assembly, as purified virions of ts48 grown at 33 °C contained a normal quantity of polypeptide VI. Similar instability of the Va polypeptide has been reported to be associated with a hexon transport negative mutant of Ad5 (Kauffman & Ginsberg, 1976). Ts48 differs from this H5ts147 mutant principally in that H5ts147 assembles the native 12S capsomere in the cytoplasm, whereas ts48 fails to achieve this trimerization.

The results of the temperature shift experiments suggested that the mutation exerts its effect continuously and apparently irreversibly, as infected cells could not be rescued by shifting down to 33 °C. Under these conditions, virtually no Va was seen and no cleavage of PVII occurred, a pattern identical to cells incubated continuously at 39 °C.

Among the pleiotropic effects of the ts48 mutation the bizarre behaviour of the fibre is notable. Although fibre polypeptides were synthesized, immunologically cross reacting fibre was reduced in the immunodiffusion test (Fig. 8a) and undetectable by immunofluorescence (Fig. 9c). Furthermore, neither 6S fibre nor 10·6S penton (fibre + penton base) could
be observed by velocity sedimentation of infected cell extracts (Fig. 11, 12). The presence of fibre polypeptides in fast sedimenting material (Fig. 10 and Fig. 12 pool 1) which resists solubilization by detergents is at present inexplicable. Such abnormal behaviour of the fibre may possibly be secondary consequences of the lack of normal assembly (trimerization) or utilization in virion assembly. Temperature sensitive mutations affecting the immunological reactivity of both hexon and fibre have been reported previously in Ad31 (Suzuki, Shimojo & Moritsugu, 1972).

Both the immunodiffusion and immunofluorescence results showed that no immunologically reactive hexon capsomeres were formed. Furthermore ts48 failed to assemble the native 360K hexon trimer antigen. In these respects ts48 resembles H5ts115, H5ts116 (Stinski & Ginsberg, 1974) and the H5ts17, H5ts20 (Russell et al. 1972; Russell, Skehel & Williams, 1974; Leibowitz & Horwitz, 1975) mutants in that all these mutants appear to be defective in immunological reactivity, hexon assembly and hexon transport. These mutants form a phenotypically distinct group compared with H5ts147 which assembles immunologically reactive hexon capsomeres that remain untransported in the cytoplasm of infected cells at the non-permissive temperature (Kauffman & Ginsberg, 1976). In addition H5ts1 and H5ts2 which have been shown to express cytoplasmic fluorescence with hexon capsomere antiserum (Russell et al. 1972) could, in view of the apparent lack of cross-reactivity of capsomere antiserum with hexon polypeptide monomers (Stinski & Ginsberg, 1974), be considered phenotypically identical with H5ts147. In spite of the phenotypic similarity between H5ts1 and H5ts2, these mutants complement and map into the 100K region and the hexon region, respectively. The electron microscopy and to a lesser extent the autoradiography results showed that ts48 infected cells accumulated virus-induced material in the cytoplasm. In view of the phenotypic resemblance of ts48 to the Ad5, ts115, ts116, ts17 and ts20 mutants, it is possible that this material comprised untransported hexon monomer polypeptides.

In view of the phenotypic similarity of ts48 to the Ad5 hexon defective mutants, the absence of complementation with any of the Ad2 or Ad5 mutants was an unexpected finding. The suspected trans-dominant nature of the ts48 mutation was confirmed in mixed infections with WT (Table 1). Results of further experiments along this line are firmly establishing the trans-dominance hypothesis. Taken together, these findings tend to argue against the possibilities that ts48 contains multiple mutations, or that it is a polar mutation. A polar mutation would be expected to show the ts effects limited to a specific class of polypeptides which map together in a single transcription unit—a situation not observed here.

Mutants with complex phenotypes such as ts48 raise the question of whether they contain more than one mutation. To rule out such a possibility is a rather difficult task. Nevertheless, we have recently isolated a revertant of ts48 which grows normally, and synthesizes a normal complement of polypeptides, but retains the thermal instability of virions which is diagnostic of ts48 (unpublished results). This result argues in favour of a single mutation in ts48.

The physical mapping of ts48 using the principle developed by Sambrook et al. (1975) is in progress. Preliminary results suggest that ts48 maps into either of two locations: that is, from 0 to 0.30 or from 0.68 to 0.76 on the unit length adenovirus genome (J. Hassell & J. Weber, unpublished data). The isolation of intertypic recombinants between ts48 and 5ts1, 5ts2, 5ts22 and 5ts36, suggests that this mutation does not lie very close to any of these Ad5 mutations. Further work is expected to clarify this ambiguity.

The pleiotropic effects of the ts48 mutation may be summarized as follows: (a) lack of
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particle assembly, (b) reduced synthesis of most infected-cell proteins, especially 95K, 80K, (c) unspecified transport defect, (d) instability of Va (Pre-VI), (e) lack of maturation cleavages, (f) aberrant immunological and sedimentation properties of IV, (g) lack of hexon capsomere polymerization.

Further work on the trans-dominant effect of the ts48 mutation is expected to help uncover the biochemical nature of this novel and previously unreported adenovirus mutation.

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