Characterization of Two Temperature-sensitive Mutants of Adenovirus Type 5

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SUMMARY

The properties of two temperature-sensitive mutants ts 18 and ts 19 of adenovirus type 5 were studied. It was demonstrated that they had a defect such that they failed to assemble virus and showed defective processing of infected cell polypeptides at the restrictive temperature. Analysis, after protease digestion, of the virions produced at the permissive temperature by SDS PAGE, and of the substrate availability of the mutants to the virus protein kinase suggested that polypeptide VI was defective in these mutants.

INTRODUCTION

A number of temperature-sensitive mutants of human adenovirus type 5 have been isolated (Williams et al. 1971; Ensinger & Ginsberg, 1972) and partially characterized genetically and biochemically (Williams et al. 1974). They appear to fall into fourteen complementation groups and have proved useful in elucidating particular aspects of virus-cell interactions (Ginsberg et al. 1974; Williams et al. 1974; Van der Vliet et al. 1975). Two of these mutants, ts 18 and ts 19, have been shown by Ustacelebi & Williams (1972) not to induce interferon in infected chick cells at the restrictive temperature. We have recently confirmed and expanded these observations and in addition have analysed the adenovirus-chick cell system using wild type and mutant viruses in an effort to understand the molecular events involved in interferon induction (Tarodi et al. 1977). On the basis of these and earlier investigations (Bakay & Burke, 1972; Ustacelebi, 1976) we have concluded that interferon induction in this system results from an early interaction between virus (or virus product) and the chick cells, and moreover that this interaction is also required for the synthesis of virus DNA in infected chick cells. Since the nature of the defect in these mutants is obviously of some relevance to the induction process we have undertaken a more detailed characterization of the ts 18 and ts 19 mutant viruses.

METHODS

Virus and cells. Preparation and purification of wild type (wt) adenovirus type 5 (Ad 75) and temperature-sensitive mutants in KB cells grown in suspension have been described in previous publications (Russell et al. 1967, 1968; Winters & Russell, 1971). Infection and labelling of infected cells were carried out in HeLa cell monolayers and the general plan of such investigations has been described before (Russell & Blair, 1977).

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Polyacrylamide gel electrophoresis (PAGE) utilized the discontinuous sodium dodecyl sulphate (SDS) buffer system after denaturation of the samples by boiling in SDS and analysis on 15% polyacrylamide gel slabs (Russell & Blair, 1977).

Virion protein kinase was determined by incubation of disrupted virus in the appropriate buffer with $^{32}$P-$\gamma$-ATP and analysis by SDS PAGE as described before (Blair & Russell, 1978).

**Enzymes.** Trypsin and chymotrypsin were obtained from Calbiochem and were of the highest quality. Beef heart cAMP-dependent protein kinase was obtained from Sigma. Digestions were carried out as described in the previous paper (Tarodi et al. 1977).

**Radiochemicals** were obtained from the Radiochemical Centre Ltd, Amersham.

**Nomenclature.** The nomenclature proposed by Ginsberg et al. (1966) for the major capsid components (hexon, penton base and fibre) is used. The remainder of the structural polypeptides are referred to according to the terminology proposed by Maizel et al. (1968) and Anderson et al. (1973) as discussed in relation to adenovirus type 5 by Russell & Blair (1977).

**RESULTS**

**Events in human cells infected with ts 18 and ts 19 viruses**

Previous results have shown that while both ts 18 and ts 19 mutants complement each other significantly (Williams & Ustacelebi, 1971) they behave very similarly with respect to the production of antigens and the synthesis of polypeptides at the restrictive temperature in infected human cells. Indeed on the basis of complement fixation and fluorescent antibody results both mutants were indistinguishable, all the major capsid antigens being detected (although in reduced amounts) with a normal distribution in infected HeLa cells (Russell et al. 1972). The analysis of polypeptide synthesis at the restrictive temperature by SDS PAGE also indicated a similar pattern of labelling to that seen in wild type infected cells (Russell et al. 1974). Thus, in contrast to other mutants, these investigations failed to detect any specific defects in ts 18 and ts 19.

These original experiments on polypeptide synthesis utilized continuous SDS PAGE systems of analysis and during the last few years the analysis of polypeptide synthesis has been significantly improved by using a discontinuous SDS PAGE system and consequently a number of other polypeptides have been recognized and described (Anderson et al. 1973; Russell & Blair, 1977). Accordingly, the pattern of polypeptide synthesis in mutant infected cells was re-examined. HeLa cell monolayers were infected with wt and mutant viruses, incubated at the permissive temperature of 33 °C and the restrictive temperature of 38.5 °C and replicate cultures were labelled with $^{35}$S-methionine for 1 h and then chased for various times from 4 to 32 h. In this way the processing of polypeptides could also be assessed by comparing the changes in the patterns of labelling during these chase periods. The labelled cell extracts were then analysed by SDS PAGE using the discontinuous buffer system and submitted to autoradiography. Fig. 1 shows the resultant autoradiograms and perusal of Fig. 1(c) will indicate the pattern of polypeptide synthesis normally seen late in infection in wild type infected cells. The changes occurring during the chase procedure are analogous in many respects to those already described for adenovirus type 2 infected cells (Anderson et al. 1973; Ishibashi & Maizel, 1974), the most prominent being the processing of the pVII polypeptide to the major core protein VII. However, other significant changes can be seen during the chase; namely, an apparent decrease in electrophoretic mobility, presumably as a function of phosphorylation, of the 72 K polypeptide (Russell & Blair, 1977); polypeptide IIIA, on the other hand, increases in electrophoretic mobility perhaps indicating some processing; a polypeptide of approximate mol. wt. 40 K also decreases in mobility perhaps again as a result of phosphorylation (Russell & Blair, 1977). A similar labelling pattern can also be recognized when analysing the mutant infected cells at the permissive
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(a) Pulse-chase analysis of infected cell extracts labelled with $^3$S-methionine. HeLa cells infected at a m.o.i. of 50 p.f.u./cell with either $ts$ t8, $ts$ t9 or wt virus at 33 or 38.5 °C were pulse labelled with 25 μCi/ml of $^3$S-methionine for 60 min at 20 h p.i. Cultures were then chased with unlabelled medium for the time indicated in the figure. The cells were harvested and subjected to SDS polyacrylamide gel analysis. The gels were dried and exposed to X-ray film. The resultant autoradiograms are shown: (a) $ts$ t8, $ts$ t9 infected at 38.5 °C; (b) $ts$ t8, $ts$ t9 infected at 33 °C; and (c) wt infected at 33 and 38.5 °C. The 72K polypeptide is the major labelled band seen between the penton base and IIIA polypeptides and the 40K polypeptide can be noted approx. midway between the fibre and 27K polypeptides.

Fig. 1. Pulse-chase analysis of infected cell extracts labelled with $^3$S-methionine. HeLa cells infected at a m.o.i. of 50 p.f.u./cell with either $ts$ t8, $ts$ t9 or wt virus at 33 or 38.5 °C were pulse labelled with 25 μCi/ml of $^3$S-methionine for 60 min at 20 h p.i. Cultures were then chased with unlabelled medium for the time indicated in the figure. The cells were harvested and subjected to SDS polyacrylamide gel analysis. The gels were dried and exposed to X-ray film. The resultant autoradiograms are shown: (a) $ts$ t8, $ts$ t9 infected at 38.5 °C; (b) $ts$ t8, $ts$ t9 infected at 33 °C; and (c) wt infected at 33 and 38.5 °C. The 72K polypeptide is the major labelled band seen between the penton base and IIIA polypeptides and the 40K polypeptide can be noted approx. midway between the fibre and 27K polypeptides.

temperature of 33 °C (Fig. 1 b). The pattern of labelling at 38.5 °C (Fig. 1 a) on the other hand, while showing no differences in the pulsed sample, clearly shows significant differences in the chased samples. Thus, there is little, if any, processing of polypeptide pVII into VII while there is no change in the mobility of polypeptide IIIA. There are, however, decreases in the mobilities of the 72K and 40K polypeptides, suggesting that phosphorylation of these polypeptides is still occurring during the chase procedures. Examination of the labelling patterns of the smaller polypeptides also suggests that some processing is defective in the mutant infected cells at the restrictive temperature.

A number of experiments were carried out with a similar format but utilizing $^{32}$P-orthophosphate (Russell & Blair, 1977) but no detectable differences could be seen in the labelling patterns (data not shown).
Studies on virus assembly

Replicate cultures of HeLa cells infected with wt and mutant viruses at 33 and 38.5 °C were labelled with ³⁵S-methionine and ³²P-orthophosphate from 8 to 48 h as described in Methods and the resultant labelled virus purified from the cells by treatment with fluorocarbon followed by caesium chloride density gradient centrifugation. On analysis of the radioactive profiles (Fig. 2) obtained from density gradients of ³⁵S-methionine labelled extracts derived from wt and ts mutant infection at 33 °C, significant radioactive peaks at virus density (1.34 g/ml) were obtained. However, the profiles obtained from labelled extracts derived from the ts mutant infection at 38.5 °C showed no labelled peak at virus density, consistent with lack of virus assembly in cells infected with the mutants at the
restrictive temperature. In a number of different experimental analyses of extracts derived from cells infected with \textit{ts} 18 and \textit{ts} 19 at the permissive temperature, it was consistently noted that two discrete opalescent bands were obtained after equilibrium density gradient centrifugation, the heavier one corresponding to virus density and the other being slightly less dense. A separation of these two species can be clearly seen in Fig. 2 on the radioactive profile obtained from extracts of cells infected with \textit{ts} 18.

\textsuperscript{32}P-labelled cell extracts gave similar results, i.e. no labelled virus could be detected at virus density in extracts derived from mutant infected cells at the restrictive temperature, while at the permissive temperature two radioactive peaks were obtained (cf. Russell \textit{et al.} 1972). On re-centrifuging the appropriate labelled fractions to equilibrium both \textit{wt} and \textit{ts}
mutant viruses re-banded at a density of 1.34 g/ml whereas the mutant infected cell extracts displayed an additional $^{32}$P-labelled peak at a density of 1.29 (Fig. 3). Investigation of these fractions by electron microscopy revealed characteristic virus particles in the wt virion peak, whereas in the virion peak derived from the mutant infected cells (at the permissive temperature), virus particles which were damaged to varying degrees and, in most cases, displaying significant clefts in the capsid, could be seen (see inserts to Fig. 3). Examination of the lower density fraction showed particles which were, for the most part, intact but appeared to be filled with negative stain suggesting the absence of virus DNA, consistent with the lower buoyant density in caesium chloride (see insert to Fig. 3). The sensitivities of the $^{32}$P label in the virions and lower density particles to treatment with proteases was determined and the results (data not shown) showed that most of the label in the virion was not rendered acid-soluble after protease digestion implying that most of the label was probably in nucleic acid. On the other hand, a large proportion of the $^{32}$P label in the low density particles was rendered acid-soluble after protease digestion suggesting that the label was principally in phosphoproteins and that the particles contained little, if any, DNA.

These studies have shown that under these conditions of virus purification, virus was not assembled in cells infected at the restrictive temperature with the ts mutants and furthermore that even the virus obtained at the permissive temperature was rather fragile and that lighter particles apparently empty of DNA could also be obtained. However, it seemed conceivable that virus particles could have been assembled at the restrictive temperature and failed to survive the extraction procedures. To test this possibility, cells infected with the mutants at both temperatures were pelleted, fixed and sectioned and the sections examined by electron microscopy. In cells infected at the restrictive temperature there were practically no particles observed and no other recognizable intermediates could be detected in contrast to those infected at the permissive temperature where many virus particles could be observed (M. V. Nermut, personal communication).

Polypeptide composition of virions

The foregoing experiments suggested that some structural component of the mutant viruses was defective, resulting in fragile particles when cells were infected at the permissive temperature. At the restrictive temperature no virus assembly occurred and the lack of processing of at least some of the infected cell polypeptides is presumably a related event. Consistent with these observations, it had previously been noted that the mutant viruses were relatively heat-labile (Ustacelebi, 1973, 1976; Tarodi et al. 1977) and in an attempt to define this structural defect further, the virions were analysed by discontinuous SDS PAGE. Coomassie blue stained electropherograms (Fig. 4) show one interesting difference between the mutant and wt viruses (slots 1 and 2). Thus, two extra polypeptides of apparent mol. wt. 19K and 11K can be discerned. These additional polypeptides were not always observed and the amount seemed to vary in different experiments. Electrophoretic analysis of the less dense particles (data not shown) indicated a lack of polypeptide VI, the core polypeptides V and VII and the smaller polypeptides X to XII. Other polypeptides were also present, their mobilities being consistent with being precursors to structural polypeptides VI and VII. This polypeptide pattern resembles that seen in 'young' virions and precursor particles (Ishibashi & Maizel, 1974; Weber, 1976) and suggests that virion assembly even at the permissive temperature is defective to some degree.

In another approach, to probe the conformation of the virion, the susceptibility of the virion polypeptides to various proteolytic enzymes was analysed. Preparations of purified wt and mutant viruses were digested under appropriate conditions with thermolysin, trypsin and chymotrypsin.

The most striking and significant differences between wt and mutant viruses were noted
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Fig. 4. Electropherograms stained with Coomassie blue of (a) purified wt and ts 18 and (b) wt and ts 19 viruses digested with chymotrypsin. Purified virus was incubated with chymotrypsin at 35 °C for 30 min at a virus/enzyme ratio (w/w) of 5.5/1. Slots: (1) wt virus, (2) purified mutant virus, (3) wt virus digested with chymotrypsin, (4) mutant virus digested with chymotrypsin and (5) chymotrypsin.

After trypsin and chymotrypsin treatment. Treatment with higher concentrations of trypsin (Tarodi et al. 1977) disrupted the virion completely digesting the core proteins and nicking the other larger polypeptides. There were differences in the patterns produced but with such extensive degradation it was not clear where the primary defect occurred. Digestion with lower concentrations of enzyme (Tarodi et al. 1977) which did not produce such extensive degradation did not reveal any difference between the wt and mutant virus preparations (data not shown). However digestion with chymotrypsin was much more illuminating. Reference to Fig. 4 shows that IIIA and III (penton base) and possibly hexon polypeptides appear to be very susceptible to enzyme degradation in both wt and mutant viruses whereas polypeptide VI in ts 18 and ts 19, but not in wt virus, is sensitive to the enzyme. This result suggests that the defect in the mutants resides in polypeptide VI although it cannot be ruled out that the susceptibility of VI to the enzyme is the result of some defect elsewhere in the virion that causes a secondary conformational change in VI making it more susceptible to protease degradation.

In vitro phosphorylation of mutant viruses

Previous work from this laboratory has shown the existence of a protein kinase tightly bound to virus particles of adenovirus types 2 and 5 (Blair & Russell, 1978). This protein kinase catalysed the in vitro incorporation of phosphate from ATP to the serine and threonine residues of polypeptides IIIA, V, VI, VII and X with IIIA being the major phosphoprotein, as demonstrated by SDS PAGE. When ts 18 virus was examined by this technique, no phosphorylation of polypeptides VI and X was detected although phosphorylation of IIIA, V and VII was normal (Fig. 5a, slot 3). Phosphorylated polypeptides of ts 19 showed the absence only of phosphorylated X – the remainder of the phosphorylated polypeptides being normal (Fig. 5a, slot 4). No thermosensitivity of the virion protein kinase of ts 18 or ts 19 could be detected in vitro.

The specificity of polypeptide phosphorylation in the ts mutants was further investigated
Fig. 5. In vitro phosphorylation of virion polypeptides of wt, ts 18 and ts 19 viruses, either by the virion protein kinase or by a cellular protein kinase. (a) Samples (50 µg) of native wild-type and mutant virions were incubated with 2 µCi γ-32P-ATP at 37 °C for 30 min in order to assay the particle-bound virion protein kinase. Phosphorylated polypeptides were analysed by electrophoresis on a linear 10 to 25 % gradient slab gel followed by autoradiography. Slots: (1) Coomassie blue stained wild-type virus, (2) wild-type virus, (3) ts 18 virus, and (4) ts 19 virus. (b) In a separate experiment, virions were heated at 90 °C for 2 min in order to inactivate the virion protein kinase. These heated virions were then used as substrate for 1 µg cAMP-dependent beef heart protein kinase, in the presence of 10 µM-cAMP. Thereafter, the assay conditions were as in (a). Slots: (1) Coomassie blue stained wild type virus, (2) wild type virus, (3) ts 18 virus, (4) ts 19 virus and (5) endogenous phosphorylation of the protein kinase preparation.

These results therefore suggest that in both mutants there are conformational changes in virion structural polypeptides which make them unavailable as substrates for the virion protein kinase. In ts 18 this is manifested as the inability to phosphorylate polypeptide VI and X, and in ts 19, polypeptide X. Since polypeptides X, XI and XII only appear in infected cells concomitant with processing of polypeptides pVI, pVII and pVIII (Weber, 1976; Fig. 2) and are absent in empty particles devoid of VI, VII and VIII, there is a possibility that polypeptide X is a derivative of polypeptide pVI.

Thus these studies provide further evidence for a defect associated with polypeptide VI or its precursor pVI.
DISCUSSION

The experiments described here have suggested that the adenovirus structural polypeptide VI is defective in both ts I8 and ts I9 mutants. A number of different observations are consistent with this suggestion, namely, (a) two minor structural polypeptides can be recognized in SDS PAGE electropherograms of mutant virus grown at the permissive temperature. Their mol. wt. are consistent with being derived from polypeptide pVI of mol. wt. 30K. (b) Digestion of the mutant viruses with chymotrypsin shows that polypeptide VI is susceptible to degradation (a situation not seen in wt virus), implying that at least the conformation of polypeptide VI in the virus particles is different. (c) Substrate availability of VI for phosphorylation by the virion protein kinase to be different in ts I8 from both wt and ts I9. These studies also showed a difference in substrate availability of polypeptide in ts I9, suggesting a relationship to VI and also consistent with the genetic evidence which suggests differences between these two mutants. (d) Marker rescue experiments (Arrand, 1978) have shown that the defect in ts I8 can be rescued by a restriction fragment which is known to contain genetic information for part of the hexon gene and the gene for polypeptide VI.

We have attempted to obtain more direct evidence of a defect in VI by tryptic peptide analysis of the polypeptide labelled in vivo with 35S-methionine and in vitro with 125I, using chloramine T (Elder et al. 1977). In all such experiments we could detect no differences in the tryptic peptide maps from wt and mutant viruses.

These studies therefore provide indirect evidence that the ts mutants ts I8 and ts I9 have a defect in a structural polypeptide VI and furthermore that this defect is sufficient to affect normal processing of some of the structural polypeptides and prevent assembly of the virus in cells infected at the restrictive temperature. The defect is also manifested at the permissive temperature since apparently defective particles are readily produced.

Polypeptide VI appears to be at least partially external on the virion since it can be readily iodinated by in vitro techniques (Rekosh et al. 1977; Everitt et al. 1975). Other studies have also suggested that it is closely contiguous with the virus core (Everitt et al. 1975).

Previous investigations (Tarodi et al. 1977) have shown that ts I8 and ts I9 infection of chick cells at the restrictive temperature failed to produce interferon and to yield late antigens and polypeptides in contrast to wt infection of chick cells, i.e. the mutants affected some ‘early’ event in infection. Thus the mutants behaved differently in human cells at the restrictive temperature, producing late components – although they failed to be assembled.

Assuming that these mutants contain only a single lesion then these experiments suggest that the conformation of the adenovirus virion is important in the induction of interferon and also has a role in the synthesis of virus DNA in chick cells. Whether the conformation per se is the critical factor in interferon induction or whether this is a sequelae of some other defect in the virion will require further study.

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