Characterization of Heavy Particles of Adeno-associated Virus Type 1

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SUMMARY

The temperature-sensitive mutant ts4 of adenovirus type 2 (Ad-2) is capable of complementing adeno-associated virus type 1 (AAV-1) in HEp2, KB and HEK cells at 34 °C and 39 °C when used as a helper virus. Heavy non-infectious AAV-1 particles can be generated by using the mutant ts4 in HEp2 cells. When AAV-1 is grown in serial passages in HEp2 cells, both the wild-type Ad-2 and the mutant ts4 give rise to heavy, less infectious AAV-1 particles. The heavy AAV-1 particles generated by Ad-2 in advanced serial passages retain the property of having CF and IF antigens, but the AAV-1 generated by the mutant in advanced serial passages lose this property. There is no appreciable difference in the particle counts made by electron microscopy of AAV-1 preparations generated either by Ad-2 or the mutant ts4. Analysis by polyacrylamide gel electrophoresis of purified heavy AAV generated by ts4 indicates that in late passage an additional polypeptide of higher mol. wt. than the three structural polypeptides is detected.

INTRODUCTION

Adeno-associated viruses (AAV) are a defective group of paroviruses, which require helper adenoviruses for productive infection (Atchison et al., 1965; Hoggan et al., 1966; Blacklow et al., 1967; Ito et al., 1967). Several temperature-sensitive (ts) mutants of adenovirus types 12 and 31 have been studied by Mayor & Ratner (1972), Mayor et al. (1977) and Handa et al. (1976), and have been shown to complement AAV antigen production as well as production of infectious virions in appropriate cells.

When lysates of cells infected with AAV types 1, 2 or 3 and the helper viruses are centrifuged through CsCl gradients a number of virus bands can be detected (Hoggan et al., 1966). The lightest band with a density between 1.32 and 1.34 g/ml consists of non-infectious AAV particles which do not contain DNA and, thus, appear empty when observed by electron microscopy (De la Maza & Carter, 1978). The major band of infectious AAV occurs at a density of 1.39 to 1.42 g/ml, and contains full genomes. The particles appear full by electron microscopy. The particles which contain complete virus genomes are also found at a higher density (Hoggan, 1971; De la Maza & Carter, 1980).

Clinton & Hayashi (1976) compared the infectivity of heavy and light particles of minute virus of mice (MVM). These populations were found to be equally infectious in mouse L-49 cells after maximum adsorption was allowed to occur (the heavy particles had a slower rate of adsorption). Also, the heavy particles could be converted easily to lighter ones by simple incubation with either infected or uninfected cell-conditioned medium.

Roy et al. (1978) reported that hyperimmune sera prepared in guinea-pigs against AAV light (L) particles (density 1.39 to 1.42 g/ml) and heavy (H) particles (density 1.46 g/ml) were antigenically different in the electron microscopic agglutination test. Anti-L serum aggregated L particles and anti-H serum aggregated H particles with maximum virus...
reactions. The average diam. of H particles also appeared to be less than that of L particles (Hoggan, 1971). Carter et al. (1979) reported that if the cells were infected with an increasing multiplicity of AAV particles banded at 1.41 g/ml in CsCl there was a corresponding decrease in production of infectious progeny of AAV. Laughlin et al. (1979) further reported that AAV (1.45 and 1.41) particles which contained standard genomes were the only infectious particles. The light density AAV (1.35 and 1.32) particles were non-infectious but interfered with the replication of heavier AAV (1.41). These particles were referred to as defective-interfering (DI) particles.

The structural polypeptides of AAV have been studied by several investigators (Rose & Koczot, 1972; Johnson et al., 1971, 1977; Sato & Mayor, 1979). It was observed that the proteins of each defective parvovirus were composed of two minor and one major polypeptide. The mol. wt. of these polypeptides were found to be similar for all three types of AAV. Furthermore, Roy et al. (1978) showed that all three structural polypeptides were present in both heavy and light virus particles of AAV type 1, although the relative amounts of two polypeptides having mol. wt. 92000 and 80000 were less in H particles as compared to the major polypeptide, mol. wt. 66000, of AAV type 3 (Johnson et al., 1971). Recently, Wild et al. (1979) have reported that the defective measles virus isolated from a subacute sclerosing panencephalitis patient was capable of synthesizing all the virus proteins with the exception of haemagglutinin when analysed by SDS–polyacrylamide gel electrophoresis (PAGE).

We have studied the complementation of AAV type 1 using a temperature-sensitive mutant ts4 of adenovirus type 2 (Ad-2) as a helper virus. The mutant ts4 was a gift from Dr J. Weber, Sherbrook, Canada. Weber and his colleagues have isolated several temperature-sensitive mutants of Ad-2 and have studied their properties extensively (Begin & Weber, 1975; Weber et al., 1975; Khittoo & Weber, 1977). The properties of the ts mutant are as follows. There is no virion assembly at the non-permissive temperature (40 °C) with ts4. Also, there is no visible band at a density of 1.34 g/ml when infected fluids are centrifuged to equilibrium in CsCl. In addition, empty virions with excessive top components appear to be defective in cleavage of certain structural polypeptides (Khittoo & Weber, 1977). Even at the permissive temperature (33 °C), the mutant ts4 accumulates more ‘top’ component than the wild-type. The top component has a buoyant density of 1.30 g/ml when centrifuged on CsCl gradients.

We report in this paper that when ts4 is used as a helper virus at both permissive and non-permissive temperatures, there is increased production of ‘heavy’ non-infectious particles of AAV with a higher buoyant density, and we describe the properties of these particles.

**METHODS**

*Tissue cultures.* HEp2 cells (human epidermoid carcinoma of the larynx), KB cells (human epidermoid carcinoma of the pharynx) and CV-1 cells (stable monkey kidney cell line) at low passage level were purchased from the American Type Culture Collection (ATCC), Rockville, Md., U.S.A. HEK (human embryonic kidney) cells were purchased from Flow Laboratories.

*Growth medium.* Cells were grown in Eagle’s minimum essential medium (MEM) (Flow Laboratories), containing 10% foetal calf serum, 0.1% sodium bicarbonate, 20 units/ml penicillin and 20 mg/ml streptomycin.

*Maintenance medium.* Growth medium which contained only 2% foetal calf serum was used to maintain infected cultures.

*Viruses.* Human Ad-2 wild-type (wt) was grown in HEp2 cells and simian adenovirus (SV15) was grown in CV-1 cells at a multiplicity of 1 tissue culture infectivity dose (TCID₅₀) per cell. Temperature-sensitive mutant ts4 of Ad-2 was grown in HEp2 cells at 34 °C. Adeno-associated type 1 virus (AAV-1) was grown either in HEp2 cells using Ad-2 as a
helper virus or in CV-1 cells using SV15 as a helper virus. The m.o.i. for AAV was 2 immunofluorescence units (IFU) per cell, and for helper adenoviruses, 1 TCID₅₀ per cell. Following a 1 h adsorption period at 37 °C, the maintenance medium was added and cultures were further incubated at 37 °C until advanced adenovirus cytopathic effects were observed. The cultures were then frozen and thawed three times, sonicated and the cell debris removed by centrifugation at 2000 rev/min for 15 min. The adenovirus was heat-inactivated at 56 °C for 20 min and the remaining AAV-containing fluid retained as stock virus.

Radiochemicals. [³H]thymidine was purchased from Schwarz–Mann Corporation, Orangeburg, New York, U.S.A. [³⁵S]methionine was purchased from Amersham International.

**Complementation of AAV-1 using ts4 as helper virus.** Monolayer cultures of HEp2, KB and HEK cells were grown on 12 mm glass coverslips in Costar wells. The cultures were co-infected with AAV-1 (free of viable adenovirus) and ts4, at 2 IFU and 1 TCID₅₀ per cell respectively. Half of the cultures were incubated at 34 °C and half at 39 °C for 1.5 h, after which 1 ml maintenance medium per well was added. At various time intervals coverslips from each set (incubated at 34 °C and 39 °C) were air-dried, fixed in acetone and stained for fluorescent antigens. The coverslips were then stained with anti-AAV-1 serum (NIH) dilution 1:640 for 30 min at 37 °C followed by several rinses with phosphate-buffered saline (PBS). The coverslips were then stained with goat anti-guinea-pig IgG fluorescein-conjugated heavy and light chains dilution 1:40 (Cappel Laboratories, Cochranville, Pa., U.S.A.) for 30 min at 37 °C followed by further rinsing in PBS. The stained coverslips were fixed on a microscope slide with a mixture of glycerol:PBS, 9:1. The coverslips were examined for positive fluorescent cells with the aid of an Ortholux II microscope (Leitz Wetzlar, F.R.G.).

**Production of infectious AAV-1 using ts4 as a helper virus.** Monolayer cultures of HEp2, KB and HEK cells in bottles were co-infected with AAV-1 and ts4 as described above. When advanced cytopathic effects were observed, the cultures were processed as described earlier.

**Immunofluorescent antigen titration of AAV-1 complemented by ts4.** Immunofluorescent antigen titrations of AAV-1 were done in HEp2 and CV-1 cells using Ad-2 and SV15 as helper viruses respectively. The cells were grown on 12 mm coverslips placed in the wells of Costar trays. The coverslips were infected with 0.1 ml per well of AAV-1 which was serially diluted tenfold in maintenance medium. A 0.1 ml amount of 1:10 Ad-2 or SV15 was inoculated per coverslip as helper viruses. The cultures were incubated at 37 °C for 24 h. At the end of the incubation period the coverslips were harvested, fixed in acetone and then stained for immunofluorescent antigen. The immunofluorescent titre of AAV-1 was determined by the method of Ito et al. (1967).

**Serial passages of AAV-1 in HEp2 cells using ts4 as a helper virus.** Monolayer cultures of HEp2 cells were co-infected with AAV-1, freed of helper virus by heating at 56 °C for 20 min just prior to use, and ts4. The cultures were divided into two groups with one group of cultures incubated at 34 °C and another at 39 °C, until the advanced cytopathic effects of ts4 were observed and then processed as described earlier.

In order to make serial passages, the AAV-1 grown using ts4 as a helper virus, was heated at 56 °C for 20 min to eliminate viable ts4. Fresh cultures of HEp2 were infected with heated AAV-1 and fresh viable ts4 as a helper source to make a subsequent passage. In this manner 10 serial passages of AAV-1 were made in HEp2 cells using ts4 as a helper virus at 34 °C and 39 °C. Ten serial passages of AAV using Ad-2 as helper were similarly made at 34 °C and 39 °C.

**Properties of AAV-1 serially passaged in HEp2 cells using ts4 as a helper.** Determination of buoyant density of AAV-1. Monolayers of HEp2 cells in 16 oz bottles were co-infected with AAV-1 in various passages, e.g. 1, 5, and 10 grown at 34 °C and 39 °C, and ts4. At 6 h post-infection, [³H]thymidine (2 µCi/ml) was added to the cultures and incubated until they
showed advanced cytopathic effects. They were frozen and thawed, sonicated and the cell debris removed by low-speed centrifugation at 2000 rev/min for 15 min. The supernatants were then subjected to high-speed centrifugation at 100,000 g for 3 h. Virus pellets were harvested, resuspended into 1/100 vol. 1 × SSC (0.15 M-NaCl, 0.15 M-sodium citrate and 0.001 M-EDTA) and sonicated to break up virus aggregates. They were then applied on 1-30 g/ml to 1-50 g/ml preformed CsCl discontinuous gradients. Gradients were spun in a Spinco SW 50.1 rotor at 35,000 rev/min at 4 °C for 18 h. Fractions were collected by bottom puncture and the ct/min of ³H determined in a Beckman LS-50 counter. The buoyant densities of AAV preparations at passage 1, 5 and 10 grown at 34 °C and 39 °C using Ad-2 or ts4 as helper were also determined. The density of each fraction was computed from refractive indices. Those fractions having densities between 1.39 and 1.49 were pooled and dialysed against 1 × SSC for 48 h. These pools were subjected to another CsCl density gradient centrifugation. The virus preparations which gave a single peak were used for studying the properties. Complement fixing antigens of AAV-1 were determined by the standard technique of Sever (1962).

Purification of AAV-1 for electrophoresis. HEp2 monolayers in 16 oz bottles were co-infected with AAV-1 in various passages (1, 5, and 10) grown at 34 °C and 39 °C, and Ad-2 or ts4, as described before. At 10 h post-infection, [³⁵S]methionine (2 μCi/ml) was added to the cultures and the incubation of the cultures was continued until advanced cytopathic effects were observed. The cultures were processed as described before, for obtaining purified virus. The lysate of the cells co-infected with AAV-1 and Ad-2 or ts4 were made as follows. After 30 h of infection the ³⁵S-labelled cells in the Petri dish were rinsed several times with PBS, then scraped off the dish with a rubber policeman. The cells were finally lysed in distilled water.

Electrophoresis and autoradiography. The PAGE system was basically that described by Laemmli (1970) and modified for slab gels by Anderson et al. (1973). The separating gels (0.75 mm thick and 160 mm × 120 mm) were formed between glass plates with a solution consisting of 7.5 % acrylamide, 0-12 % N,N'-methylenebisacrylamide (BIS), 0-1 % SDS, 0-5 M-urea, 0-7 % ammonium persulphate, 0-03 % N,N,N',N'-tetramethylethylenediamine and 0-375 M-tris-HCl pH 8-8 to 9. The stacking gel consisted of 4 % acrylamide, 0-1 % SDS, 0-5 M-urea, 0-7 % ammonium persulphate, 0-1 % BIS, 0-125 M-tris–HCl pH 6-6 and 18 % sucrose. The remaining procedures were similar to those described by Salo & Mayor (1979).

RESULTS

Table 1 shows the infectivity titres in HEK cells of the mutant ts4 grown in HEp2, KB and HEK cells, at permissive and non-permissive temperatures. The results were read 10 days post-infection. As can be seen, there is very little leak at the non-permissive temperature. Table 2 shows the immunofluorescent (IF) titres and complement fixing (CF) antigen titres of AAV-1 complemented by the mutant ts4 in comparison to the wild-type at permissive and non-permissive temperatures. The production of IF and CF antigens of AAV-1 by ts4 was higher in HEp2 cells than those in KB or HEK cells at both temperatures. Therefore, we decided to use HEp2 cells for further studies. At the non-permissive temperature the mutant showed an ability to produce IF antigen well, but not CF antigens. Table 3 presents the properties of AAV-1 produced using Ad-2, wt and ts4 as helper viruses at permissive and non-permissive temperatures following serial passages in HEp2 cells. Several observations can be made. (i) The buoyant density of the majority of AAV-1 particles increased following serial passages at both temperatures, by using Ad-2 or ts4 as helper viruses. The buoyant density increased from 1.42 to 1.47 when ts4 was used as a helper. (ii) The production of CF antigens decreased as serial passages advanced, even at permissive temperature, when ts4 was used as a helper source. (iii) By using ts4 as a helper the production of AAV-1 IF antigens in
Table 1. Infectivity titres TCID<sub>50</sub>/ml of the mutant ts4 in HEK cells at 34 °C

<table>
<thead>
<tr>
<th>Cells*</th>
<th>34 °C</th>
<th>39 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEp2</td>
<td>6.1</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>KB</td>
<td>4.8</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>HEK</td>
<td>4.1</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Cells in which ts4 was grown.

Table 2. Antigenic properties of AAV-1 complemented by ts4

<table>
<thead>
<tr>
<th>Source of AAV-1*</th>
<th>CF antigen titre</th>
<th>IF titre per ml (log&lt;sub&gt;10&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HEp2 cells using Ad-2 helper†</td>
</tr>
<tr>
<td>HEp2 at 34 °C</td>
<td>16</td>
<td>4.9</td>
</tr>
<tr>
<td>HEp2 at 39 °C</td>
<td>2</td>
<td>4.7</td>
</tr>
<tr>
<td>KB at 34 °C</td>
<td>8</td>
<td>4.5</td>
</tr>
<tr>
<td>KB at 39 °C</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>HEK at 34 °C</td>
<td>4</td>
<td>4.4</td>
</tr>
<tr>
<td>HEK at 39 °C</td>
<td>4</td>
<td>4.0</td>
</tr>
<tr>
<td>HEp2 at 34 °C</td>
<td>32</td>
<td>6.0</td>
</tr>
<tr>
<td>HEp2 at 39 °C</td>
<td>16</td>
<td>6.2</td>
</tr>
<tr>
<td>HEp2 at 37 °C using Ad-2 as a helper</td>
<td>32</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* AAV-1 stocks were obtained by co-infecting HEp2, KB and HEK with AAV-1 and ts4 at 34 °C and 39 °C. After advanced cytopathic effects were observed the cells were harvested, frozen and thawed, sonicated and centrifuged. The stocks were inactivated at 56 °C for 20 min before use.

† IF antigen titrations of the AAV stocks were carried out in HEp2 and CV1 cells using Ad-2 and SV15 as helper viruses respectively, at 37 °C. The infected cells were fixed 24 h after incubation for IF staining.

HEp2 cells decreased following serial passages. At 39 °C AAV-1 antigens were not detectable subsequent to the second passage, and at permissive temperature (34 °C) they disappeared after the 4th passage. This effect was not shown when Ad-2 wt was used as a helper. (iv) There is a dramatic decrease in IF antigen production in the purified 100× concentrated AAV-1 preparations made after 10 passages. There is a marked reduction in the IF antigen titre when the mutant ts4 was used as helper, as compared to that of wt adenovirus. The IF antigen titre of AAV-1 using Ad-2 wt as a helper at passage 1 is log<sub>10</sub> 8.3 and log<sub>10</sub> 8.7 at 34 °C and 39 °C respectively, while those at passage 10 are log<sub>10</sub> 6.8 and log<sub>10</sub> 6.6. The difference in IF antigen titres between passage 1 and passage 10 was about 100-fold. In comparison when ts4 is used as a helper this difference was approx. 10000-fold.

(v) Although there was a marked reduction in infective AAV-1 virus as assayed by IF antigen production, there was no appreciable difference in the particle counts of these preparations.

Fig. 1 shows the results of analysis by PAGE of the structural polypeptides of AAV-1 grown at 34 °C and 39 °C using ts4 as a helper virus. The major structural polypeptide VP3 of AAV-1 in the cell lysate overlaps the fibre protein of the adenovirus (lane a). Lane (a) shows the position of adenovirus hexon (H) and the expected positions of AAV-1 proteins VP1, VP2 and VP3. The bands are at the expected mol. wt. of 92000, 88000 and 66000. Lane (c) represents the analysis of polypeptides prepared from purified AAV-1 at passage 1 at 34 °C. All three structural polypeptides VP1, VP2 and VP3 are clearly resolved. In addition, there is evidence for the presence of some adenovirus hexon (H). Lane (e) represents the polypeptides of AAV-1 preparation at passage 5 at 34 °C. Besides all four polypeptides—three of AAV-1 (VP1, VP2, VP3) and the contaminating H—there is an extra protein (E) with
Table 3. Properties of AAV-1 produced by using Ad-2 and ts4 as helper viruses in serial passages in HEp2 cells

<table>
<thead>
<tr>
<th>Source of AAV-1 (passage no.)</th>
<th>Buoyant density*</th>
<th>CF antigen titre/ml</th>
<th>IF antigen titre/ml (log₁₀)</th>
<th>IF antigen titre/ml (log₁₀)</th>
<th>Particle ct/ml‡</th>
<th>Purified concentrated virus†</th>
<th>Buoyant density*</th>
<th>CF antigen titre/ml</th>
<th>IF antigen titre/ml (log₁₀)</th>
<th>IF antigen titre/ml (log₁₀)</th>
<th>Particle ct/ml‡</th>
<th>Purified concentrated virus†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-1/Ad-2 (1)</td>
<td>1.41</td>
<td>32</td>
<td>6.4</td>
<td>8.3</td>
<td>5.4 x 10⁹</td>
<td>1.42</td>
<td>16</td>
<td>6.8</td>
<td>8.7</td>
<td>6.1 x 10⁹</td>
<td>1.44</td>
<td>ND</td>
</tr>
<tr>
<td>AAV-1/ts4 (1)</td>
<td>1.43</td>
<td>8</td>
<td>4.6</td>
<td>6.3</td>
<td>5.1 x 10⁹</td>
<td>1.42</td>
<td>4.5</td>
<td>6.4</td>
<td>4.0 x 10⁹</td>
<td>3.0 x 10⁹</td>
<td>1.47</td>
<td>ND</td>
</tr>
<tr>
<td>AAV-1/Ad-2 (10)</td>
<td>1.44</td>
<td>ND†</td>
<td>&lt;2</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>4.3 x 10⁹</td>
<td>1.44</td>
<td>ND†</td>
<td>4.8</td>
<td>6.6</td>
<td>1.5 x 10⁹</td>
<td>ND†</td>
</tr>
<tr>
<td>AAV-1/ts4 (10)</td>
<td>1.46</td>
<td>&lt;2</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>4.3 x 10⁹</td>
<td>1.47</td>
<td>&lt;2.0</td>
<td>1.5 x 10⁹</td>
<td>3.0 x 10⁹</td>
<td>1.47</td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Buoyant densities in CsCl were estimated by plotting ct/min against refractive indices of AAV-1 preparations labelled with [³H]thymidine; only virus preparations which gave single peaks were used.
† The virus was purified and concentrated (100×) for IF antigen titrations and particle counts.
‡ Particle counts were made according to the method of Smith & Melnick (1962).
§ ND, Not done.
Fig. 1. Autoradiogram of electrophoretically separated proteins produced during AAV-1 and Ad-2 or ts4 co-infections at either 34 °C or 39 °C temperatures. Proteins were labelled with 2 µCi/ml [35S]methionine 10 h post-infection. Purified AAV-1 preparations were analysed in 7.5% polyacrylamide slab gels. Lane (a), lysate of HEp2 cells co-infected with AAV-1 and Ad-2 at 34 °C. Lane (b), purified AAV-1 preparations grown at 37 °C using Ad-2 as a helper (old preparation having fewer 35S ct/min). Lane (c), purified AAV-1 preparations grown at 34 °C using ts4 as a helper. Lane (d), purified AAV-1 preparations grown at 39 °C using ts4 as a helper. Lane (e), purified AAV-1 preparations grown at 34 °C (passage 5) using ts4 as a helper. Lane (f), purified AAV-1 preparations grown at 39 °C (passage 5) using ts4 as a helper. Lane (g), purified AAV-1 preparation grown at 34 °C (passage 10) using ts4 as a helper. H, adenovirus hexon; E, 'extra' protein.

a mol. wt. of about 100000. Lane (g) represents the polypeptide pattern of AAV-1 at passage 10 using ts4 as a helper at 34 °C. It is clear that the E protein appears to persist in later serial passages of AAV-1 preparations.

Very faint bands of VP3 in identical positions are resolved in AAV-1 preparations grown at 39 °C using ts4 as a helper at passages 1 and 5 (lanes d and f respectively). AAV-1 grown at 39 °C using ts4 as helper at passage 5 shows the presence of E protein (lane f) which is absent in passage 1 (lane d). VP3 is also faintly visible in an old preparation of purified AAV-1 grown using Ad-2 as a helper at 37 °C (lane b). The concentration of 35S label in the AAV preparations grown at 34 °C using ts4 was higher than in those grown at 39 °C, and as a result, polypeptides resolved from 39 °C preparations are faint (lanes d and f). In spite of this, the extra E protein is clearly resolved in the preparation of AAV made at 39 °C at passage 5 (lane f).

**DISCUSSION**

AAV is a defective virus which requires the presence of a helper virus (either adenovirus or herpesvirus) for its replication. Co-infection of cells with AAV and a helper adenovirus results
in production of virus particles with different densities in CsCl (Hoggan et al., 1966). The majority of infectious AAV particles have a buoyant density of 1.41 g/ml while the dense particles having 1.45 g/ml buoyant density are physically unstable and less infectious. Furthermore, Roy et al. (1978) have reported that the light and dense particles of AAV are antigenically different in the electron microscopic agglutination test. Although the capsids of the dense particles are composed of the same three structural polypeptides as that of 1.41 g/ml particles, the concentrations of the two minor proteins have been reported to be less than the dense particles. However, a later study has not confirmed this finding (De la Maza & Carter, 1978).

The mutant of human adenovirus type 3, ts13, which is deficient in its DNA replication at the non-permissive temperature, is capable of complementing AAV growth (Ito & Suzuki, 1970; Mayor et al., 1977). This suggests that the replication of the DNA of the helper virus per se is not mandatory in the replication of AAV. Myers & Carter (1980) have studied the assembly of adeno-associated virus by pulse-chase labelling experiments and concluded that DNA is not required for the assembly or structural integrity of the AAV protein capsid.

The multifaceted defectiveness of AAV has been discussed by Young & Mayor (1979). It appears that AAV represents an extreme case of virus defectiveness in that it must rely on a helper virus at each stage of its replication. Of particular interest, was the need for a helper function for the assembly of AAV structural proteins into an antigenic form which was still distinct from the mature virus capsids (Young & Mayor, 1979).

Recently, Carter et al. (1980) have shown that in human KB cells adenovirus mutants ts125 or ts107, having temperature-sensitive lesions in the E72 DNA binding protein coded by the adeno early region 2, are deficient for AAV growth at 40 °C. Using these mutants, they observed that the accumulation of AAV particles as well as AAV capsid proteins was decreased without an appreciable decrease in DNA synthesis. Thus, the early region of the adenovirus gene appears to play a positive role in transcription or post-transcriptional processing of some AAV RNAs. Ostrove & Berns (1980) studied AAV replication using various early and late ts mutants of adenovirus as helper viruses and observed that an adenovirus function or functions, in addition to those required for AAV DNA replication is needed for AAV rescue from latently infected Detroit 6 cells. Both these studies concern the defectiveness of AAV DNA replication rather than the maturation steps.

In our studies using mutant ts4, we were able to produce dense AAV particles which presumably did not contain a full complement of DNA. The dense particles are less infectious than the 1.41 g/ml particles. This is in agreement with the findings of Roy et al. (1978). Also, Clinton & Hayashi (1976) have demonstrated that the dense particles of MVM appeared to be less infectious because of their slow rate of adsorption to the host cells. They also showed that all three classes of proteins were found in light and heavy particles but their proportions varied.

In addition to the three virion structural proteins, other AAV-specific proteins have previously been identified in infected cells by PAGE. Johnson et al. (1977) reported as many as eight specific AAV-induced polypeptides, ranging in size from 56,000 to 120,000 daltons. They consequently presented a model based on post-translational cleavage of the 120,000 dalton protein into the three structural proteins. Conversely, Buller & Rose (1978) and Salo & Mayor (1979) were not able to identify any AAV-1-specific proteins larger than those found in the virion. Buller & Rose (1978), however, found two small proteins which appear to be AAV-specific. These investigators also reported that post-translational cleavage might be occurring to yield the three structural polypeptides. In their model, cleavage of the largest structural polypeptide results in the production of the two smaller ones.

Previous studies by Richardson et al. (1980) have indicated that the early adenovirus mRNA can specify all the functions necessary for AAV growth. A recent report by Janik et
al. (1981) has identified four 'early' regions of the adenovirus type 2 genome which may be needed to support replication of AAV. These regions are the early region 1A mapping near the left-hand terminus of the Ad-2 genome, early regions 2 and 4 mapping from 59.5 to 79.9 and 89.7 to 100, respectively and the so-called virion-associated VA I RNA mapping from 28.5 to 29.4. The VA RNA region is of particular interest because although its synthesis begins early in infection (within 3 h) it is much more abundant late in infection. VA RNAs are not spliced, capped or polyadenylated and do not appear to have an mRNA function. Because of their position near the 3' end of a long stretch of structural genes it has been suggested that VA RNAs might play a role in transcriptional regulation and processing and alignment sites for mRNA splicing (Mathews, 1980). They could play a similar role for AAV mRNA, whose nucleotide sequences around the spliced junctions show some homology with corresponding regions in both VA I and VA II RNAs (Green & Roeder, 1980). Adeno 2 mutant ts4 maps in the region of the VA RNAs and of virion proteins IIIa and V (penton) (Hassell & Weber, 1978). Thus, changes in VA RNA could be important in the processing of AAV structural proteins.

Our preliminary polypeptide analysis by PAGE of purified AAV-1 generated by ts4 indicates that in late passage an extra polypeptide with a higher mol. wt. than the three structural polypeptides is present. This presents additional evidence that there is indeed a precursor for AAV coat protein.

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