Electron Microscopy of Adenovirus Cores

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(Accepted 4 March 1975)

SUMMARY

Adenovirus type 5 ‘cores’ prepared by heating in the presence of deoxycholate and partially purified on a glycerol density gradient could be visualized as roughly isometrical particles with a condensed centre from which twisted filaments or loops of DNA emanated. This compact structure was readily dispersed by spreading on distilled water or by treatment with EDTA, Nonidet, DNase or trypsin. Spreading with Nonidet was particularly effective in unfolding the cores and revealing long filaments about 100 Å thick presumably of the virus nucleoprotein. Subunits (about 30 to 60 Å in diam.) could be seen free in the DNase-treated cores, suggesting a particulate nature of one or both of the core proteins.

INTRODUCTION

Human adenovirus type 5 contains a single, linear double-stranded DNA and at least five distinct polypeptides (van der Eb, van Kesteren & van Bruggen, 1969; Russell et al. 1973). Three of these polypeptides are associated with the capsid components, hexon, penton base and fibre, while at least two are internal and associated in some way with the DNA (Russell, McIntosh & Skehel, 1971). The cores prepared by heating in the presence of sodium deoxycholate (Russell et al. 1971) are the subject of this ultrastructural analysis. The aim of this work was to characterize the cores using different techniques of electron microscopy and to study the effects of various treatments on their integrity. In the long term, in parallel with a more biochemical approach, it is hoped to come to a better understanding of the disposition of the basic core components along the virus DNA and to ascertain if they have some properties other than structural.

METHODS

Preparation of purified virus cores and virus DNA. Human adenovirus type 5 (strain Ad 75) was grown in HeLa or KB cells and purified by fluorocarbon extraction of the infected cells followed by one velocity gradient and two equilibrium density gradient sedimentations in caesium chloride. The purified virus was disrupted by treatment with 0·5 % sodium deoxycholate (DOC) at 56 °C for 1 to 2 min, and the cores separated from other virus components by centrifuging in a 10 to 40 % glycerol gradient in 5 mm-tris/HCl, pH 7·6 (Russell et al. 1971). Those fractions containing cores as determined by u.v. absorption (Russell et al. 1971), were either dialysed against 5 mm-tris/HCl buffer, pH 7·6, or used for electron microscopy after suitable dilution with PBS or distilled water.

The virus DNA was prepared either from purified virus (after heating at 56 °C for 1 min)

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or from cores by treatment with pronase at 37 °C for 1 h followed by sedimentation through a pad of caesium chloride of density 1.50 g/ml as previously described (Mäntyjärvi & Russell, 1969). The clear, gelatinous DNA pellet was gently resuspended in 5 mM-tris/HCl buffer, pH 7.6, and extracted with tris buffer-saturated phenol (van der Eb et al. 1969). Phenol was removed by dialysis of the combined aqueous extracts against dilute tris/HCl buffer. In some cases the initial pelleting of DNA was omitted and the pronase-treated extract was extracted with phenol. The virus DNA had $E_{260}/E_{280} > 1.85$.

**Reagents.** Except as noted below, standard reagent grade chemicals from commercial supply houses were used without further purification. Nonidet P-40 was from BDH (Poole, England). Pancreatic deoxyribonuclease I (DNase) and bovine trypsin, both once crystallized and lyophilized, were from Sigma London Chemical Co. (Kingston-upon-Thames, Surrey, England). DNase was dissolved in 5 mM-tris/HCl buffer, pH 7.6 (dilute tris/HCl), and used at a final concentration of about 50 µg/ml, with or without 10 mM-MgCl₂. Trypsin was dissolved in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), and used at final concentrations of 10 to 50 µg/ml in a mixture of PBS, 5 mM-tris buffer and 20% glycerol. Soybean trypsin inhibitor was obtained from Worthington Biochemical Corp. (Freehold, New Jersey, USA) dissolved in PBS, and used at the same concentrations and in the same solutions as the trypsin. Pronase was obtained from Sigma London Chemical Co., heat-treated and stored in 1.0 M-NaCl at pH 7 as described by Kates & Beeson (1970), and used at a final concentration of 300 µg/ml in 20% glycerol and 5 mM-tris/HCl buffer.

**Electron microscopy.** The cores either straight from the glycerol gradient or after dialysis were applied to carbon-coated parlodion films (on 400 mesh grids) or to plain carbon films (i) by adsorption from a drop, followed by washing on distilled water, (ii) by spreading on distilled water or 0.3 M-ammonium acetate, pH 8.0 (Kleinschmidt et al. 1962), followed by washing with distilled water and negative staining, positive staining with alcoholic uranyl acetate, and rotary shadowing, respectively. In a typical experiment 0.05 ml of virus cores were mixed with 0.01 ml of 0.1% cytochrome c (in 0.1 M-ammonium acetate, pH 7.0) before spreading (Nermut, 1972). In another experiment 0.05 ml of cores was mixed with 0.01 of 0.1% Nonidet P-40 and, after 1 min at room temperature, spread on distilled water.

The following techniques were used for visualization of the cores by electron microscopy: (a) negative staining with 3% ammonium molybdate, pH 6.5, or 4% silicotungstate, pH 6.5, or 1% uranyl acetate, pH 4.4; (b) positive staining with alcoholic uranyl acetate (10⁻⁴ M in acid ethanol) according to Li & Seto (1971); (c) rotary shadowing with uranium oxide at an angle of about 10°; (d) freeze-drying 'on the grid' as described by Nermut, Frank & Schäfer (1972) with Pt-C shadowing at an angle of about 40°.

Specimens were examined under a Philips EM-300 electron microscope operating at 60 kV. Plates of Pt-C shadowed 'cores' were reversed (to give dark shadows); the rotary shadowed ones were not reversed.

**RESULTS**

The general appearance of cores differed from preparation to preparation and appeared to be influenced by the mode of preparation for electron microscopy. Preparations made by adsorption showed roughly spherical compact bodies with filamentous protrusions which were either scarce or very abundant depending on the DOC treatment. Shorter treatment resulted in compact cores with a few filaments but with many contaminating capsomers, longer treatment in looser cores with many filaments in form of loops or twisted 'arms' and only a few capsomers. Fig. 1 illustrates the cores seen with negative staining; another batch of cores even when stained with uranyl acetate looked not quite so compact. The negative
stain used also influenced the appearance of cores. Their structure was looser and coarser after ammonium molybdate (Fig. 2) or silicotungstate than after uranyl acetate which revealed a very fine granular surface different from the background of the micrograph (Fig. 3). It is of course difficult to decide what contribution the phase contrast, the supporting film and the negative stain make to this appearance. However it seems likely that the surface of the cores is not smooth and that the size of the ‘granules’ observed may be about 30 to 40 Å. It was also noted that after prolonged storage at 4 °C fairly loose cores with long strands were seen, suggesting presence of filamentous structures in the cores (Fig. 4). The thickness of such filaments varied from 70 to 100 Å or more.

A striking difference was observed between specimens prepared by adsorption and those
Fig. 3. High magnification of a core from the same preparation as in Fig. 1, showing a fine granular surface structure.

Fig. 4. Cores dialysed against 0.005 M-tris buffer, pH 7.6, overnight, adsorbed on to the grids and negatively stained with uranyl acetate.
Fig. 5. Cores prepared by adsorption and rotary-shadowed. Note their dense centres with only a few filaments around them.

Fig. 6. Same as above but prepared by spreading on distilled water. Note the total absence of dense centres.

Fig. 7. Similar preparation as in Fig. 6 but stained with alcoholic uranyl acetate which binds preferentially to DNA, but may also stain proteins.

Fig. 8. Same as in Fig. 7 but spread on distilled water. The cores are much less electron-dense which may be caused either by relaxation (flattening) or by loss of some protein.

Fig. 9. Cores disintegrated into clusters of minute granules following treatment with 50 µg/ml DNase at 20 °C for 5 min. No DNA filaments were revealed in such preparations. Negative staining with uranyl acetate.
prepared by spreading. The dense, compact centres seen after rotary shadowing or positive staining in specimens prepared by adsorption (Fig. 5 and 7) mostly disappeared or became less dense after spreading on distilled water (Fig. 6 and 8). Similar effects were obtained after suspending the cores in 1 mM-EDTA for 10 min; addition of Mg-salts to the medium did not cause any marked change in the core structure.

Two questions arose in connection with the 'arms' and 'loops' emanating from the cores (Fig. 5 to 8); what is their nature and are they native or preparation artifacts? We attempted to answer the first question by treating the cores with DNase. This was carried out either in a suspension (at 37 or 20 °C) or on an electron microscope grid (at 20 °C) and the incubation times varied from 1 to 20 min. In a control experiment the cores were treated with PBS containing 10 mM-Mg²⁺ (PBS + Mg). The loops disappeared after 1 min treatment with DNase but the cores were still compact particularly when treated on the grid and at 20 °C. Prolonged treatment (more than 5 min) led to relaxation or even to disintegration of the cores into smaller 'islands' or groups of minute granules measuring about 30 to 60 Å (Fig. 9). The 'loops' remained intact after treatment with PBS + Mg for 20 min at 37 °C, but the cores were dispersed to some extent. The thickness of the loops was measured on positively stained as well as rotary-shadowed preparations and compared with the thickness of purified virus DNA prepared in the same way at the same time. The figures obtained were about 20 Å for positively stained filaments and 80 to 100 Å for shadowed ones both in cores and DNA. These results together with those obtained after DNase digestion suggest that the loops were of free DNA and not a nucleoprotein complex.

It is generally known that air-drying from aqueous solutions causes many drying artifacts due to surface tension forces (Anderson, 1954), and it was therefore considered necessary to visualize cores with one of the techniques which prevent drying artifacts, to rule out the possibility that the loops were liberated from the cores by the conventional preparatory techniques. We used the freeze-drying technique and found that the cores were fairly high irregular bodies, but the loops were not revealed by unidirectional shadowing with Pt-C (Fig. 10). However, when the freeze-dried specimens were rotary-shadowed at a low angle the loops were clearly seen (Fig. 11), suggesting that they were present before the preparation for electron microscopy. Presumably they were liberated from the cores following DOC treatment, as their frequency varied from batch to batch and as they were not noted after mechanical disintegration of virus (M. V. Nermut, unpublished results).

In an attempt to gain some idea of the fine organization of the DNA and protein in the cores we tried other methods of loosening and unfolding the internal structure. As already noted, spreading had some effect on the cores but did not unfold them entirely, and therefore the effect of a mild detergent, Nonidet P-40, was examined. Most cores were further relaxed by this treatment and a complex network of strands was often observed (Fig. 12). The thickness of the strands varied from 70 to 120 Å, but very thin filaments (presumably DNA) emanated from these structures suggesting that some portions of DNA were free of protein as a result of DOC or Nonidet treatment. The thicker strands revealed a granular substructure but this was not as distinct as after the DNase treatment. The effect of a proteolytic enzyme on the organization of the core was also examined. The cores were treated with trypsin as described in Methods for intervals of 20, 40 and 80 min. Appropriate controls were carried out with simultaneous observations on untreated cores, to which a preincubated mixture of trypsin and trypsin inhibitor were added, and finally purified DNA for comparison of strand thickness. The cores were substantially loosened during the first 20 min and this process proceeded further. Fig. 13 shows a general appearance of cores after rotary shadowing. The thin long filament running across the micrograph (presumably DNA)
Fig. 10. Cores prepared by adsorption and freeze-dried. Shadowed with Pt-C at an angle of about 40° and reversed. The cores stand high, but hardly any loops are seen.

Fig. 11. Same as in Fig. 10 but rotary-shadowed at about 10° after freeze-drying. The loops are now well seen.

Fig. 12. Cores mixed with Nonidet (0.02% final concentration), spread on distilled water and negatively stained with uranyl acetate.

Fig. 13. Cores treated with trypsin (10 μg/ml) for 20 min, spread on distilled water and rotary-shadowed. Note the sausage-like formations compared with the long thin filament presumably DNA (arrows).
measures 80 Å in diameter, whereas the strands are 110 to 200 Å thick (no correction has been made for metal shadowing). Thus this treatment suggested that proteins did have some part to play in the constraint applied to the DNA in the core structure.

**DISCUSSION**

Combination of different preparatory techniques showed that the ‘DOC-cores’ are three-dimensional bodies with an electron-dense centre and twisted filaments or loops of DNA emanating out of them. This basic morphology varied from batch to batch, and cores without any loops as well as fairly relaxed cores were observed in different preparations. This suggests that the DNase-sensitive loops were liberated from the cores primarily by the DOC-treatment and should therefore be regarded as preparation artifacts. The freeze-drying technique showed that loops were not liberated from the cores by surface-tension forces during drying. On the other hand the cores were fairly relaxed (losing their dense centre) after spreading on distilled water as well as after treatment with EDTA or a low concentration of Nonidet P-40. Similarly Robinson, Younghusband & Bellett (1973) described release of closed loops of DNA from chick embryo lethal orphan virus particles treated with urea-perchlorate or simply stored for several weeks and spread on a water surface. All these findings suggest that the DNA-loops are very easily liberated from the virus particles under adverse conditions. One can speculate that at least a portion of the DNA is either randomly packed inside the core without any specific interactions with the two core-proteins or that its specific association with the core-proteins is very weak. Cores relaxed with EDTA or Nonidet or treated with trypsin are still relatively constrained, indicating that some protein is still present.

Electron micrographs of cores stained negatively with uranyl acetate showed a fine granular surface and the cores were fairly compact. Conversely, negative staining with silicotungstate or ammonium molybdate revealed relatively loose structures where distinct but coarser granularity could be discovered. Similar pictures were published earlier by Russell, Valentine & Pereira (1967) after heat-treatment, by Laver et al. (1968) after acetone and by Russell et al. (1971) after DOC-treatment. Unfortunately, no uranyl-acetate-stained micrographs were published by these authors to compare them with the present ones. The above difference in the core appearance can be accounted for by a higher penetrability of uranyl acetate (Haschemayer, 1970) and by its stabilizing effect on biological structures in contrast to a fairly deleterious effect of the salts of phosphotungstic acid on biological macromolecules or membranes (Glauert & Lucy, 1969; Muscatello & Horne, 1968). High-resolution electron micrographs of the more relaxed cores following treatment with either Nonidet or DNase showed the presence of small spherical or cylindrical particles (approx. 30 Å in diam. or up to 60 Å long) which could constitute a complex of either one or both of the core-proteins.

It has been reported by Itzhaki & Cooper (1973) that in chromatin from different sources 42 to 47% of the DNA-phosphate groups are available for binding poly-lysine, which means that only about 50 to 60% of the DNA-phosphate groups bind to the histones. As the adenovirus nucleoprotein is very basic it may bind to DNA-phosphates in a similar way as the histones. Furthermore, the particulate nature of the DNA-associated protein would fit into such a concept. In some respects the adenovirus cores show morphological similarities to the compact and relaxed DNA complexes associated with disrupted *Escherichia coli* (Worcel & Burgi, 1972), and with isolated chromatin components (Hamkalo & Miller, 1973; Comings & Okada, 1974). These similarities presumably reflect the nature of the folding of
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the DNA, since in all these cases the DNA has to be constrained into a relatively small volume overcoming considerable electrostatic repulsive forces, presumably with the aid of basic proteins and/or other cationic components. The core structures seen in this study also suggest that at least some of the DNA is packaged within the virus particle in the supercoiled form, showing some similarities with the supercoiled circular DNA molecules packaged within papovavirus particles (Weil & Vinograd, 1963). In the case of adenovirus the presumed supercoiling of the DNA would be constrained by the virus core proteins, suggesting a more specific role for these proteins in contrast to the papovaviruses where the circularity of the DNA molecule provides the necessary constraint, and the evidence suggests that the core proteins are not virus-specific and are derived from the histones of the host cells (Fey & Hirt, 1974). Further studies will be required and are in progress to elucidate the internal organization of the virus particle.

The able technical assistance of Mrs Barbara Ward, Mrs Carol Newman and Mr John Wills is greatly appreciated. One of us (JAH) gratefully acknowledges support of the US Public Health Service through Research Career Development Award no. GM-14252, and a leave of absence granted by Case Western Reserve University, Cleveland, Ohio, for pursuing this investigation.

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(Received 6 January 1975)