Characterization of a New Adenovirus Type 5 Assembly Intermediate

By MAURICE L. J. MONCANY,* BERNARD RÉVET and MARC GIRARD†

Unité de Physiologie des Virus,
Institut de Recherches Scientifiques sur le Cancer,
B.P. N° 8–94800 Villejuif, France

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SUMMARY

Adenovirus H5 (Ad5) DNA–protein complexes were extracted with ammonium sulphate (0.2 M) from virus-infected HeLa cell nuclei at 18 h after infection. Analysis of the material by centrifugation through discontinuous sucrose gradients in heavy water revealed the existence of several populations of molecules which were identified, in order of increasing buoyant density, as mature DNA–protein complexes, replication complexes, assembly intermediates and virions. When observed under the electron microscope, some of the assembly intermediates showed a capsid with a tail of entirely double-stranded (ds) DNA, or of dsDNA continued by a portion of single-stranded (ss) DNA thickened by a coat of E-72 K DNA binding protein. Singly or doubly-forked Ad5 replicating DNA molecules partially packaged in virus capsids were also observed. It is suggested that these molecules could be assembly intermediates, i.e. one of the first steps of assembly corresponding to virus DNA entering pre-formed capsids or their precursors. The fact that replication was still going on at one end of many of the DNA molecules in the intermediates, while encapsidation was taking place at the other, raises the possibility of a coupled DNA replication-packaging process in the formation of adenovirions.

INTRODUCTION

The assembly of adenoviruses (Ad) is still a poorly elucidated process. This is partly due to the complex organization of the virion, which is made of no less than nine structural proteins assembled in a 252 capsomere icosahedral capsid, containing the \(23 \times 10^6\) mol. wt. virus DNA (for review, see Philipson & Lindberg, 1974). Two internal proteins, proteins V and VII (Anderson et al. 1973; Everitt et al. 1973) are supposed to hold the DNA molecule in a compact form inside the capsid (Laver, 1970; Prage & Pettersson, 1971; Brown et al. 1975). Several discrete assembly intermediates have been described (Ishibashi & Maizel, 1974; Edvarsson et al. 1976; Weber, 1976; Windberg & Wadell, 1977; D'Halluin et al. 1978a, b) and it has been established that the formation of virions results from the penetration of newly replicated virus DNA molecules into pre-formed capsids or their precursors.

* Present address: Biozentrum der Universität, Basel, Department of Microbiology, Klingelbergstrasse, 70, CH-4056, Basel, Switzerland.
† To whom reprint requests should be addressed.
Little is known, however, about the mechanism by which the 12 \mu m long virus DNA molecule can enter a capsid with a diameter of only 70 to 80 nm, or about the possible relationship between this process and replication of the DNA molecules (for review, see Winnacker, 1978).

It was first shown by Wilhelm et al. (1976) that brief incubation of Ad-infected cell nuclei in 0.2 M-ammonium sulphate resulted in the release of most of the virus DNA replication and transcription complexes in an active form, together with mature virus DNA–protein complexes and virions. In the course of fractionating such nuclear extracts by equilibrium centrifugation in discontinuous sucrose–heavy water gradients we have discovered a population of molecules which appear to be intermediates between replicating virus DNA molecules and virions and which, most likely, represent DNA molecules penetrating into virus capsids by one of their ends; some of these DNA molecules seem to be still undergoing replication at the other end. It is suggested that these molecules could represent one of the early steps of encapsidation. A model which might account for their formation is presented.

**METHODS**

**Infection and labelling.** HeLa cells grown in Eagle's suspension medium supplemented with 10 % foetal calf serum were infected with Ad5 at 50 to 100 p.f.u./cell as previously described (Girard et al. 1977). Labelling of the cells was with either 200 \mu Ci/ml \textsuperscript{3}H-thymidine (40 Ci/mmol: CEA, Saclay, France) added at 17.75 h p.i. (in a ten times concentrated cell culture), or with 0.05 \mu Ci/ml \textsuperscript{14}C-thymidine (53.9 Ci/mmol: CEA), added at 15 h p.i. Incorporation was stopped at 18 h p.i. by pouring the labelled cultures on to frozen phosphate-buffered saline (PBS) containing 10 mM-EDTA and 1 mM-unlabelled thymidine. Proteins were labelled with 20 \mu Ci/ml \textsuperscript{35}S-methionine (623 Ci/mmol; CEA) added from 9 to 14 h p.i. in a medium containing 1/10th the normal concentration of methionine and 2 % dialysed calf serum, after which the cells were centrifuged and resuspended in complete medium with 10 % serum. Incorporation was stopped as above.

**Preparation and fractionation of nuclear extracts.** The cells were washed twice with ice-cold PBS containing 10 mm-EDTA and nuclei were prepared as described by Wilhelm et al. (1976) except that the concentration of EDTA in the buffers was 10 mm. Nuclei were resuspended in 20 mm-tris-HCl, pH 7.9, 1 mm-DTT and 0.2 % Triton X-100, then made 0.2 M with respect to ammonium sulphate. After a 10 min incubation in ice, with occasional homogenization using a loose pestle in a Dounce homogenizer, the nuclei were centrifuged for 10 min at 10,000 g. The supernatant was made 400 \mu g/ml with heparin (Choay, Paris, France) and centrifuged through a discontinuous sucrose gradient extemporaneously prepared by layering, on top of one another, 3 ml of a sucrose cushion with a density of 1.28, 5 ml of a similar cushion with a density of 1.24, 6 ml with a density of 1.20; 4 ml with a density of 1.16, 4 ml with a density of 1.12 and 4 ml with a density of 1.08. Each of the layers was in 20 mm-tris-HCl, pH 7.9, 1 mm-EDTA, 200 mm-NaCl, 1 mm-spermidine, 1 mm-DTT and 0.2 % Triton X-100 (TENSDT buffer) and was prepared with heavy water (CEA). The density of each solution was controlled by weighing known volumes using micropipettes. The gradients were centrifuged for 7 h at 25,000 rev/min and 4 °C, in the SW27 Spinco rotor, after which 0.6 ml fractions were collected with an ISCO fractionator and analysed for acid-precipitable radioactivity, DNA polymerase activity and protein content, before being pooled as indicated in the legend to the figures.

**Determinations of endogenous DNA polymerase activity.** Aliquots of the fractions from the discontinuous sucrose gradients were incubated for 30 min at 37 °C in the presence of
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40 mM-tris-HCl, pH 7·9, 5 mM-MgCl₂, 3 mM-DTT, 100 mM-ammonium sulphate, 50μM each of dATP, dCTP and dGTP, and 5μM-³H-dTTP (500 to 700 ct/min/p mol), in a final vol. of 100μl. The reaction was stopped by the addition of 0·1 M-NaOH, after which the acid insoluble radioactivity in the samples was determined. Analysis of protein by SDS-PAGE was as described by Laemmli (1970), except that the gels were 7·5 to 15% polyacrylamide gradient slab gels. Electrophoresis was for 5 or 6 h at 80 V and then gels were stained with Coomassie blue, destained and processed for fluorography (Laskey & Mills, 1975). Phosphorylase A (94 K mol. wt.), bovine serum albumin (68 K mol. wt.), ovalbumin (45 K mol. wt.), chymotrypsin (25·5 K mol. wt.) and cytochrome c (12·4 K mol. wt.) were used as markers.

Electron microscopy. The material to be examined was dialysed against 10 mM-tris-HCl, pH 7·9, 1 mM-EDTA, 100 mM-NaCl, at 4 °C, before being prepared for electron microscopic observation according to the technique of Dubochet et al. (1971) and shadowed with platinum.

Miscellaneous. For determination of buoyant densities in CsCl, the material was prefixed overnight with 5% neutral glutaraldehyde as described by Baltimore & Huang (1968) and 0·25 ml were layered on top of 5 ml pre-formed CsCl gradients from a density of 1·45 to 1·70 g/ml. The gradients were centrifuged for 5 h at 35000 rev/min and 20 °C in an SW50.1 Spinco rotor. Determination of buoyant densities in metrizamide was as previously described (Girard et al. 1977) using pre-formed metrizamide gradients with a density range from 1·056 to 1·3080 in heavy water and computing densities (ρ) from refraction indexes (η) following the equation \[ ρ = 3·0917 × η - 2·9984 \] (Rickwood & Birnie, 1975). Acid-precipitable radioactivity was determined by collecting the precipitates formed by the addition of 5% trichloroacetic acid on glass fibre filters (GF/C Whatman) and counting these in a toluene-based scintillation mixture. In some experiments, radioactivity was directly assayed by counting aliquots of the fractions in the toluene-based mixture diluted with 0·5 vol. Triton X-100.

Analysis of deproteinized DNA molecules at neutral or alkaline pH was as previously described (Girard et al. 1977).

RESULTS

Isolation of DNA–protein complexes

Ad5-infected HeLa cells were labelled with ¹⁴C-thymidine at 15 h p.i. then with ³H-thymidine at 17·75 h and a nuclear extract was prepared at 18 h p.i., using 0·2 M-ammonium sulphate (Wilhelm et al. 1976). The extract was analysed by sucrose gradient centrifugation in a discontinuous gradient in heavy water (see Methods). Analysis of acid-precipitable radioactivity in the gradient revealed the presence of several populations of nucleoprotein complexes, which were fractionated on the basis of their density as indicated in Fig. 1(a). Average densities were 1·08, 1·12, 1·18, 1·20 and 1·24 for fractions A to E, respectively. Most of both the ³H label (open circles) and the ¹⁴C label (triangles) was recovered in fractions A, B and C. In addition, a variable proportion of the ¹⁴C counts was recovered as a peak in fraction E, which corresponded to complete virions (see below). The fact that ³H and ¹⁴C labels show similar profiles while ¹⁴C represents mainly mature molecules and ³H those which are replicating, proves that it is necessary to shorten the ³H pulses. Moreover, with ³H-thymidine labelling, a part of the incorporation of radioactivity could, in part, be due to repair-DNA synthesis and not to replication. In view of this, by using shorter ³H pulses in recent experiments, the labelled material was deproteinized and analysed by sucrose gradient centrifugation at either neutral or alkaline pH. The results, not presented here, showed that
Fig. 1. Fractionation of extracts from Ad5-infected cell nuclei by centrifugation through discontinuous sucrose gradients in heavy water. Nuclear extracts were prepared from Ad5-infected HeLa cells either (a) doubly labelled with $^3$H and $^{14}$C-thymidine or (b) labelled with $^{35}$S-methionine as described in Methods. The extracts were centrifuged through discontinuous sucrose gradients in heavy water and the resulting fractions were assayed for acid precipitable $^3$H (○—○), $^{14}$C (△—△) or $^{35}$S (●—●) radioactivity respectively. Centrifugation was from left to right. Fractions from the gradient in (b) were also assayed for incorporation of $^3$H-dTMP in the presence of all four deoxyribonucleoside triphosphates (○—○). The brackets labelled A to E refer to the fractions which were pooled for further analysis.

the $^{14}$C label was in mature virus DNA molecules, whereas the $^3$H label was partly in mature DNA molecules and partly in incomplete (nascent) DNA molecules.

A similar fractionation experiment was performed using nuclear extracts from $^{35}$S-methionine-labelled infected cells. The majority of the $^{35}$S label was found in the fractions above the gradient, i.e. in soluble proteins (Fig. 1b, closed circles). In addition, two peaks of labelled material were clearly visible, one in fractions A and B, and the other in fraction E.

Since it is known that extraction of Ad-infected cell nuclei with 0.2 M-ammonium sulphate recovers virus replication complexes almost quantitatively (Brison et al. 1977; Kedinger et al. 1978), identification of the active replication complexes in the gradient of Fig. 1(b) was attempted. This was done by assaying for deoxyribonucleoside triphosphate-dependent in vitro incorporation of $^3$H-dTMP in each of the fractions from the gradient. As shown by the open circles in Fig. 1(b), the majority of the endogenous DNA polymerase activity was associated with the material sedimenting in fractions A and B, and a smaller proportion with that sedimenting in fractions C and D, suggesting that active DNA replication complexes were present in the corresponding regions of the gradient.

Analytical polyacrylamide gel electrophoresis

The nature of the $^{35}$S-labelled proteins associated with the different populations of molecules was determined by polyacrylamide gel electrophoresis (PAGE) on part of the material from the gradient in Fig. 1(b). PAGE–SDS analysis of the material in fractions A and B
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Fig. 2. Samples from fractions A to E from the gradient in Fig. 1(b) were analysed by electrophoresis through a 7.5 to 15% polyacrylamide gradient slab gel as described in Methods and the labelled material was revealed by fluorography. The figures 72, 63, 43 and 23, referring to the K mol. wt. of the corresponding polypeptides, were estimated by comparison with the electrophoretic mobility of known markers, shown stained with Coomassie blue in the left hand lane of the figure, and with that of 35S-methionine-labelled adenovirion polypeptides (not shown). Nomenclature for structural polypeptides follows that of Ishibashi et al. (1977).

revealed the presence of four major bands of labelled polypeptides (Fig. 2) with apparent mol. wt. of approx. 72 K, 63 K, 50 K and 43 K, respectively. Densitometry scanning of the gel showed that the majority of the label was in the 72 K band. Also, this band was the only one visible after Coomassie blue staining (not shown). It probably corresponds to the early virus-coded, DNA-binding protein E-72 K (Van der Vliet & Levine, 1973; Van der Vliet et al. 1975, 1977; Levine et al. 1976), which has been shown to be the principal protein constituent of the adenovirus DNA replication complexes (Kedinger et al. 1978; and our unpublished observations). The protein is attached to the ssDNA portions of replicating virus DNA molecules.

PAGE-SDS analysis of the material in fractions C to E from the discontinuous sucrose gradient revealed a progressive change in the polypeptide composition of the fraction (Fig. 2). Thus, the lower the position of the material in the sucrose gradient, the lower its content in non-structural adenovirus polypeptides (as, for example, the 72 K, 50 K, 43 K mol. wt. bands) but the higher its content in virus capsid polypeptides [as, for example, polypeptides II (hexon), III (penton-base) or IV (fibre) and the low mol. wt., hexon-associated poly-
peptides VI, VIII and IX or their precursors]. The content of each fraction in the internal virion polypeptide VII (or its pVII precursor) also seemed to follow that of the structural capsid polypeptides: pVII and VII were absent in fractions A to C, barely detectable in fractions D, but clearly evident in fraction E. Internal virion polypeptide V, which was also clearly seen in fraction E, was present at a much lower level in fraction D; but it is uncertain whether or not it was absent from fractions A and B in view of the possible confusion with the polypeptide band at about 50 K (labelled V in Fig. 2). There was no visible band of histones in the gels, contrary to what was observed in the nuclear pellet from the same cells (results not shown). Indeed, it is known that Ad DNA is not complexed with histones and that there is a rapid shutdown of histone synthesis in Ad-infected cells (Kit & Daniell, 1978).

**Electron microscopy**

The fact that all, or almost all of the known virus structural proteins were recovered in fraction E, together with mature virus DNA, suggested that the material in that fraction was made up of complete virions. This was demonstrated by electron microscopic analysis after negative staining (results not presented), and by showing that purified adenovirions banded at a similar density (1.24 g/ml) when sedimented in parallel in a discontinuous sucrose gradient in heavy water.

The material from the gradients in Fig. 1 was next prepared according to Dubochet et al. (1971) and examined under the electron microscope. This spreading method allows ds or ssDNA covered by proteins to be observed.

The aspect of the material from fractions A, B and C, under the electron microscope was that of fibrillar tangles, irregular in size, apparently resulting from the multimolecular association of two kinds of DNA molecules, one with the aspect of thin filaments, approx. 6 to 7 nm in diam. (ds DNA) and the other with that of thick filaments, 13 to 14 nm in diam. (ssDNA covered by proteins). Identical observations have been made by Kedinger et al. (1978) on the Ad2 DNA replication complexes, for which they showed that the thick filaments corresponded to ss virus DNA fully coated with the E-72 K DNA binding protein. A similar interpretation probably applies here in view of the fact that, as shown above, the major protein detected in fractions A to C was the 72 K mol. wt. protein, and that these fractions contained the majority of the endogenous DNA-polymerase activity of the nuclear extract (Fig. 1 b).

Fractions A and B probably correspond to simple Ad5 DNA replication complexes and also to mature DNA–protein complexes, as could be shown by zonal centrifugation of the fractions (results not presented). An additional component appears to be part of fraction C, since examination of the material in that fraction under the electron microscope reveals the presence of dark coloured structures with the shape and size of virus capsids. Consistent with this observation is the fact that large amounts of hexon polypeptide II are also present in fraction C (Fig. 2). The possibility exists, therefore, that the material in fraction C represents one of the first stages of the encapsidation of newly made virus DNA or binding of capsids to DNA. At times, the observation of isolated DNA molecules in fraction C (Fig. 3) permitted their shape to be followed and showed images of displacement synthesis (forked dsDNA molecules with a single stranded arm) or of duplication synthesis (unforked dsDNA molecules terminated by a single-stranded segment) as observed with deproteinized molecules (Ellens et al. 1974; Lechner & Kelly, 1977) except that the ssDNA regions of the molecules were covered by proteins.

Under the electron microscope fraction D was seen to contain material one step further ahead in the evolution from DNA replication complexes towards virions. Tangles disappeared and isolated molecules were much easier to find. The micrographs in Fig. 4, illustrating two of the major aspects of these molecules, show that they were made of a
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Fig. 3. Isolated DNA molecules in C fractions from the discontinuous sucrose gradient in Fig. 1(b). The material was prepared according to the method of Dubochet et al. (1971). ds, Double-stranded DNA; ss, single-stranded DNA.

capsid (on average 76-61 nm ± 1.39 nm wide) which seemed to be attached to the end of either a fully dsDNA molecule of variable length (Fig. 4c, d and e) or of a segment of dsDNA, continued by a variable length of protein-covered ssDNA (Fig. 4a and b). These kinds of images could be interpreted as resulting from either DNA packaging or DNA release. The presence of ssDNA, however, supports the first hypothesis, as does the observation of capsid-linked singly forked molecules (Fig. 5a) or doubly forked molecules (Fig. 5b), corresponding to known images of DNA displacement synthesis (Lechner & Kelly, 1977; Winnacker, 1978).

For these reasons and for ease of notation, we propose to call the population of isolated DNA molecules with a virus capsid attached at one end, as found in fraction D, 'previrions 1', and the population of mixed replicating DNA molecules and capsids found in fraction C 'previrions 2'.

Density determinations

Pulse-chase experiments should help to confirm that these complexes are true previrions. Their density in CsCl after fixation with glutaraldehyde was 1.309 g/ml (Table 1), indicative of a high protein content. When analysed on a metrizamide gradient, 'previrions 1' were
Table 1. Densities of Ad5 virions and 'previrions'*

<table>
<thead>
<tr>
<th></th>
<th>Virions</th>
<th>'Previrions 1'</th>
<th>'Previrions 2'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose-heavy water</td>
<td>1.24</td>
<td>1.20</td>
<td>1.16-1.18</td>
</tr>
<tr>
<td>CsCl</td>
<td>1.346</td>
<td>1.309</td>
<td>1.338</td>
</tr>
<tr>
<td>Metrizamide</td>
<td>1.236</td>
<td>1.213</td>
<td>1.196</td>
</tr>
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* The material from fractions C ('previrions 2'), D ('previrions 1') or E (virions) from the gradients in Fig. 1 was concentrated by centrifugation on to a sucrose cushion with a density of 1.32 g/ml in TENS/DT buffer prepared with heavy water, after which aliquots of the samples were either dialysed or diluted to the required density and their buoyant density was determined (see Methods).

heavier than 'previrions 2', confirming their higher degree of proteinization. The difference between 'previrions 1 and 2' was best demonstrated by centrifuging them through a CsCl solution of density 1.72 g/ml in the absence of glutaraldehyde fixation; whereas 'previrions 1' did not penetrate the gradient, thymidine label associated with 'previrions 2' was found to band at the position of Ad DNA. This shows that 'previrions 2' were dissociated by the high ionic strength of the CsCl solution, suggesting that they are labile intermediates. On the other hand, the non-penetration of 'previrions 1' into the gradient suggest that they remained intact during centrifugation and therefore do not result from artefactual binding of capsids to virus replicating or non-replicating DNA molecules. This conclusion can also be reached from the fact that they behaved as a homogenous population when recentrifuged in sucrose-heavy water, or in metrizamide gradients (Table 1).

**Significance of previrions**

The length of the free, visible, portion of the DNA molecules in 'previrions-1' was usually much shorter than that of the virus genome. This is presumably due to the fact that the remaining part of the DNA molecule was already packaged inside the capsid. Fig. 5(a) and (b) show replicating molecules with a capsid at one end of the dsDNA. If the DNA molecules in these micrographs were indeed in the process of encapsidation by one of their ends (that attached to the capsid) while still undergoing replication at their other end (the free end), the direction of the replication forks on the molecules should be unequivocally towards their free end (arrows in Fig. 5). This, in turn, allows the length of the still unreplicated portion of the DNA molecule to be measured, from which it is easy to estimate the expected length the displaced ssDNA arms should assume. For example, the percentage of replication of the DNA molecule in Fig. 5(a) can be computed as 90% [i.e. (12-1.1):12; for measurements, see the legend to Fig. 5]. It follows that the length of the ssDNA arm in that micrograph should have been approx. 10.9 \( \mu m \) (the exact figure depending upon the degree of condensation imparted to the DNA molecule by the attachment of the E-72 K protein) instead of the observed 2.38 \( \mu m \). Similarly, the length of the left hand side ssDNA arm in Fig. 7(b) should have been approx. 9.85 \( \mu m \) (equal to the length of dsDNA presumably already wrapped inside the capsid, added to the length of the DNA segment between

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Fig. 4. Isolated assembly intermediates in fraction D from the sucrose gradients in Fig. 1(b). The material was prepared according to the method of Dubochet et al. (1971). Micrographs (a) and (b) show partly ss and partly dsDNA molecules. In (b), phage PM2 was used as a marker for measuring the thickness of dsDNA. The arrow labelled ss indicates the single-stranded DNA and the arrow labelled ds indicates the double-stranded DNA. The ssDNA portion of the molecule in (a) is 4.43 \( \mu m \) long and the dsDNA segment 0.07 \( \mu m \) long. In (b) the ssDNA part is 2.19 \( \mu m \) long, and the dsDNA sequence 0.25 \( \mu m \). Micrographs (c), (d) and (e) show dsDNA molecules. The length of DNA is 8.22 \( \mu m \) in (c), 2.55 \( \mu m \) in (d) and 4.34 \( \mu m \) in (e).
Fig. 5. Aspects of 'previrions' with branched DNA molecules. (a) Singly forked molecule; (b) doubly forked molecule. The arrows indicate the branching points and the likely directions of the corresponding replication forks. The material was prepared according to Dubochet et al. (1971). In (a) the ssDNA is 2.38 μm long and the overall length of the dsDNA portion is 4.10 μm (3 μm between the capsid and the fork, and 1.1 μm between the fork and the free end of the molecule). In (b), the first ssDNA arm is 2.20 μm long and the second one 1.43 μm long. The total length of the dsDNA portion is 4.18 μm, distributed as follows: 2.03 μm between the capsid and the first fork, 0.71 μm between the two forks and 1.44 μm between the second fork and the free end of the molecule.
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Fig. 6. (a) Samples from fractions C, D and (b) E from the gradient in Fig. 1(b) were centrifuged through alkaline (pH 13) 5 to 20% sucrose gradients as described by Girard et al. (1977). The resulting fractions were assayed for acid-precipitable $^3$H-radioactivity present in 'previrions 2' (fraction C, △——△), 'previrions 1' (fraction D, □——□), virions (fraction E, ●——●). A 24S DNA corresponds approximately to half a genome.

the capsid and the replication fork) and not 2.20 μm as observed. The discrepancy between the expected figures and those actually measured shows that either the packaging contained incomplete DNA molecules, or that the ssDNA portions of the molecules were more fragile and were accidentally cleaved during the extraction and processing of the samples.

To investigate this question, the DNA in 'previrions 1 and 2' and in virions was analysed by sucrose gradient centrifugation in denaturing conditions at pH 13 (Fig. 6). Whereas the majority of the virions were shown to contain 34S adenovirus DNA, most (60%) of the DNA in 'previrions 1 and 2' sedimented at 24 to 26S, corresponding to half genome length and a minor part at 34S. D'Halluin et al. (1978a, b) have previously reported the existence of Ad2 encapsidation intermediates containing a 7 to 11S virus DNA fragment, but the relationship between those intermediates and previrions, although suspected, has not been established.

As many of the replicative intermediates linked to capsids appear as tangles, it is difficult to quantify the different types of structures observed (furthermore, the ratio of replicative DNA is only less than 5% of the total virus DNA extracted from infected cells). Most of the time, the isolated complexes were dsDNA of variable length linked to a capsid; similar structures were also observed by Kedinger et al. (1978). Isolated complexes with single-stranded tails were rare, as well as forked complexes. Forked molecules are also rare among pure replicative intermediates.
Nuclear extracts prepared from Ad5-infected cells were fractionated by centrifugation in discontinuous sucrose gradients in heavy water in the presence of 0.2 M NaCl. This method allowed separation, from top to bottom of the gradients, of soluble nuclear proteins, mature virus DNA-protein complexes, virus DNA replication complexes, 'previrions 2' (a mixture of DNA replication complexes and of capsids), 'previrions 1' (isolated virus DNA molecules in the process of encapsidation at one of their ends while still possibly undergoing replication at the other end), and virions. 'Previrions 1 and 2' could be differentiated from one another by their appearance under the electron microscope and by their respective buoyant densities. 'Previrions 1' were stable in CsCl whereas 'previrions 2' were not.

Analysis of the proteins in these different molecular complexes suggest that the 'previrion-step' could be a key step in the packaging of virus DNA. At this step, the non-structural proteins (such as 72 K, 50 K or 43 K mol. wt. proteins) disappear, whereas the structural proteins (hexon, penton-base, fibre, etc.) appear and/or become preponderant. However, 'previrions' are incomplete particles, both because the DNA molecules they are encapsidating are still partly hanging loosely out of the particles and because, compared to virions, they show a partial absence of internal proteins V and VII. The addition of internal polypeptide VII (or its pVII precursor), in the last few steps leading to the formation of virions, has already been reported (Levine et al. 1974; D'Halluin et al. 1978a, b), but remains a matter of controversy. If, indeed, protein VII plays a crucial role in the compacting of the DNA inside the mature adenovirion, it is hard to imagine how the incoming DNA molecule could wrap itself inside the capsid in the absence of that protein. It should also be pointed out that no protein VII or pVII was detected in the fractions containing purified mature or replicating Ad DNA molecules. These molecules appeared as essentially naked when examined under the electron microscope (see Fig. 5). Very occasionally, a few nucleosomes seemed to be present on mature Ad DNA molecules (Fig. 4c and e), but this is probably due to artefactual leakage of nucleosomes from cell chromatin at the time of extraction, as demonstrated by Kedinger et al. (1978). The conclusion that ds Ad DNA molecules appear to be naked inside the infected cell can also be drawn from the studies of Corden et al. (1976) and from the electron micrographs of Matsuguchi et al. (1979). As shown here, Ad dsDNA molecules entering virus capsids also assume a well spread appearance, with no evidence for association with protein (Fig. 4 and 5). This leads to the conclusion that either the protein(s) (or small mol. wt. molecules) responsible for compacting of the virus DNA were lost during the extraction and processing of the samples, or that compacting takes place only at the site of entry of the virus molecule into the pre-formed capsid (or a precursor of it) and even perhaps only after it has entered the capsid.

The apparent density of 'previrions 1' was 1.309 in CsCl after fixation with glutaraldehyde. This is to be compared with the results obtained by Ishibashi & Maizel (1974), Windberg & Wadell (1977), Edvarsson et al. (1976) and D'Halluin et al. (1978a) who described intermediate adenovirus particles with a density of 1.30 to 1.306 g/ml. The new property which was found here for at least some of these particles is that they contain an associated DNA-polymerase activity and that the DNA they are packaging can still be undergoing replication on the end opposite that which is encapsidated (Fig. 4a, b; Fig. 5). These results suggest a possible scheme for the replication and encapsidation of Ad DNA which is depicted in Fig. 7 and according to which a replicating DNA molecule could be sucked into a pre-formed capsid (or a precursor of it) by one end while still replicating on the other end.

Temporal and physical coupling of the replication and encapsidation of virus genomes has been previously suggested to exist in the case of parvoviruses (Tattersall & Ward, 1976) and of different bacteriophages (Yamagishi & Okamoto, 1978). The possible coupling between
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Replicating DNA molecules

Capsids

'Previrions'

Virions

Fig. 7. A possible model for the encapsidation and replication of Ad DNA molecules. The model shows how encapsidation into a pre-formed capsid could take place on the left end of a molecule the right end of which was still undergoing replication. The molecules of terminal protein (Rekosh et al. 1977) at the 5' ends of the DNA strands have been represented by a dot. This model is independent of the possible concomitant maturation steps of the proteins in the capsid (Anderson et al. 1973; Everitt et al. 1973).

Ad DNA replication and encapsidation which is suggested by this scheme should be easy to test. This could be done in pulse-chase experiments using ts mutants as, for example, the E-72 K DNA binding protein mutants described by Ensinger & Ginsberg (1972) or mutants for which the replicating molecules continue their elongation but which cannot initiate new synthesis. Other mutants, such as the encapsidation mutants described by Weber (1976), Edvarsson et al. (1976, 1978) or D'Halluin et al. (1978a, b) could help to determine whether the DNA is packaged in a capsid (or a capsid-precursor) or if the shell is organized at the initiation extremity of the genome. In these experiments, the utilization of restriction enzymes could answer the recurrent question which is whether, in the case of adenoviruses, encapsidation of DNA can start at both ends of the molecule, at random, or, as suggested by Daniell (1976) for Ad3, only on the left end of the genome.

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