JOURNAL OF VIROLOGY, Dec. 1979, p. 995-1005
0022-538X/79/12-0995/11$02.00/0

Vol. 32, No. 3

In Vitro Association of Empty Adenovirus Capsids with Double-Stranded DNA

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Received for publication 30 April 1979

Several lines of evidence suggest that empty adenovirus capsids are preassembled intermediates in the pathway of virion assembly. We have observed that purified empty capsids of subgroup B adenoviruses have a remarkable affinity for DNA in vitro. The products of capsid-DNA association are sufficiently stable, once formed in low-salt solution, to permit purification and characterization in CsCl density gradients. Neither virions nor the DNA-containing incomplete particles of subgroup B adenoviruses can give rise to such in vitro reaction products. The average molecular weight of the empty adenovirus capsids is about 123×10^6, consistent with the absence of viral core peptides and a small deficiency of exterior shell polypeptides. Electron microscopy of negatively stained capsids and the capsids bound to DNA reveals a typical adenovirus size and architecture. The particles appear with a surface discontinuity that is presumed to expose the DNA binding site(s). The DNA molecules associated with the empty capsids are susceptible to the actions of DNase and restriction endonucleases. The dependence of rate of capsid-DNA association on DNA length suggests randomly distributed binding sites on the DNA molecules. Although the DNA molecules can successively acquire additional empty capsids, the empty particles themselves are restricted to interaction with only one DNA molecule. Electron microscopy of the capsid-DNA complexes spread in cytochrome *c* films shows that the particles are bound along the contour of extended duplex DNA. The amount of DNA within each bound particle appears to be less than 300 base pairs, as estimated by the length of the DNA molecules visible outside of the bound particle. The empty capsid-DNA association product described in this report provides an interesting substrate for further investigation of the DNA packaging process in a defined in vitro system, with extracts or purified components from infected cells.

The ultimate product of adenovirus genome expression during productive infection is a massive accumulation of mature virions among the remains of the infected cell. The structures of these virus particles are complex icosahedral shells, about 80 nm in diameter, comprised of at least 12 distinct viral polypeptides. Each particle encloses a 23×10^6-dalton double-stranded viral DNA genome. In its highly condensed form, the virion DNA is associated with basic polypeptides V and VII (11), as a core structure reminiscent of the nucleoprotein organization of chromatin (5). The driving forces and precise order of DNA-protein interactions which lead the newly replicated viral DNA to assume the compacted form associated with product virions are not yet known. Similarly, little is known regarding mechanisms of DNA packaging for other DNA-containing viruses and bacteriophages (16). Several lines of evidence now implicate empty adenovirus capsid structures, lacking DNA and basic core polypeptides, as preassembled substrates for DNA packaging in vitro. The precursor-product relationship of empty capsids to virions is supported by pulse-chase labeling experiments focused on their respective structural proteins (14, 21). However, the large pools of viral polypeptides which accumulate in various stages of capsid assembly do interfere with analysis of these experiments. Ambiguities regarding the structural stability of various intermediates can obscure their status in the normal pathway of virion assembly (6, 9). This is also a problem for using mutants blocked in assembly of virions under restrictive conditions (7, 10). Attempts have been made to avoid disruptive purification procedures or to stabilize intermediates of adenovirus assembly (6, 9).

Examination of the physical properties of packaged DNA and its relationships with asso-

ciated proteins of the capsid is another approach to study mechanisms involved in the DNA packaging process. This already has been pursued in a variety of bacteriophage and animal virus systems (2, 3, 5, 8, 12, 18). General consideration also has been given to the mechanical and thermodynamic aspects of viral DNA encapsidation (19).

A complementary approach is based on developing and studying in vitro DNA packaging systems. Lysates from cells infected by adenovirus assembly mutants or infected under conditions which block virion assembly have been used (27, 28). Such systems, however, have a serious drawback due to the variety of events which must proceed to produce infectious virions or interpretable abortive assembly intermediates. Selective consideration of a specific aspect of the overall process, such as DNA packaging, is difficult under such circumstances.

We have considered the abundant pool of empty viral particles purified from subgroup B human adenovirus-infected cells as a potentially useful material for in vitro study of interactions related to the process of virion assembly. These particles have a remarkable affinity for double-stranded DNA molecules in vitro, giving rise to stable products of association, which we describe in this paper. The simplicity of this system and the well-defined course of the interaction permit concentration on physical properties related to the DNA packaging process. The nature of the system further invites evaluation of the transfection potential of various DNA molecules packaged in vitro and administered at high multiplicity to susceptible animal cells in culture.

MATERIALS AND METHODS

Cells and viruses. Adenovirus type 3 (Ad3, strain G.B.) and type 7 (Ad7, strain Gomen) were propagated in suspension cultures of HeLa S3 cells as described previously (25).

Virus and phage DNA. Adenovirus DNA was extracted from purified virions by treatment with pronase B and Sarkosyl and by extraction with phenol, chloroform, and isoamyl-alcohol and was then centrifuged in isopycnic CsCl density gradients (25). Labeling of adenovirus-infected cells with [^{32}P]orthophosphate and preparation of radioactive DNA was performed as previously described (26). Phage lambda DNA (strain $\lambda cI_{857}Sam_7$) was a generous gift of Helios Murialdo, University of Toronto. The λDNA was dialyzed against 0.01 M Tris-hydrochloride (pH 8) and 0.001 M EDTA and heated to 80°C just before use to reduce concatemers to monomer-length species. DNA from phage φX174, RF-I, was purchased from Bethesda Research Laboratories, Bethesda, Md. Conditions for restriction enzyme digests of viral and phage DNA and the physical maps of restriction sites in Ad3 and Ad7 DNA were presented earlier (23).

Empty adenovirus capsids. Infected cell extracts were centrifuged twice to equilibrium and fractionated to select for the uppermost of the Ad3 or Ad7 top component particles (corresponding to the pool IP-k in the lower panel of Fig. 1 in Tibbetts [24]). This material was diluted with four volumes of 0.1 M Tris-hydrochloride (pH 8) and layered onto preformed, 10-ml linear CsCl gradients from a density of 1.2 to 1.4 g/ml. After centrifugation for 60 min in an SW41 rotor (35,000 rpm at 4°C), the predominant opalescent band of particles was recovered, diluted with 1.3-g CsCl/ml, and centrifuged to equilibrium as before. The concentrated empty adenovirus capsids purified in this manner were minimally contaminated by residual incomplete particles. Such preparations were stored in 2.4 M CsCl at 4°C and showed no aggregation or change in their capacity to interact with DNA in vitro over many months. The concentration of empty adenovirus capsids was estimated by absorbance at 260 nm in the presence of 0.5% sodium dodecyl sulfate. Under these conditions virions (13% DNA) are estimated to be 10^{12} particles per ml per unit of absorbance; empty particles are about 4×10^{12} particles per ml per unit of absorbance.

Gel electrophoresis. DNA samples were subjected to electrophoresis in 1% agarose slab gels (0.3 by 28 cm) at 40 V by using 0.09 M Tris, 0.09 M boric acid, and 0.001 M EDTA buffer. [^{32}P]DNA was detected in the gels by autoradiography by using Kodak RP-XOMAT or XRP-1 X-ray films.

Electron microscopy. Samples of adenovirus particles for negative staining were absorbed onto Formvar-coated copper grids, fixed with 4% glutaraldehyde, washed, and then stained with 2% uranyl acetate, pH 4.6. DNA-empty capsid complexes from density gradients were diluted with 4 volumes of 0.2 M ammonium acetate, fixed briefly with 0.5% glutaraldehyde, mixed with cytochrome *c* (0.2 mg/ml), and immediately spread onto a hypophase of 0.15 M ammonium acetate. Formvar-coated grids were used to pick up portions of the protein film from the surfact of the hypophase. Grids were rinsed with 90% ethanol and stained with uranyl acetate as described above.

Density gradients. Samples representing reaction mixtures of empty capsids and DNA were centrifuged in CsCl density gradients with initial density of 1.32 g/ml. Fractions were collected either dropwise after puncture of the bottom of the tube or as equal volumes from the top of the gradient by pumping through a flow cell monitor to a fraction collector. Absorbance measurements through the flow cell were made at 254 nm. Refractive index (25°C) determinations were made on the collected samples immediately after fractionation of the gradients, for use in calculating values of density through the gradient (4). In some experiments it was necessary to separate fragments of free DNA from fragments still in association with empty capsids. The background contamination of small fragments of free DNA was eliminated by adjusting the sample to 1.5-g CsCl/ml (1 ml), overlayering with 1.3-g CsCl/ml (3 ml), and centrifuging this step gradient overnight at 40,000 rpm in an SW56 rotor. These conditions permit particles to float up into the lower density region of the gradient, together with particle-bound DNA, while the free DNA sediments from no

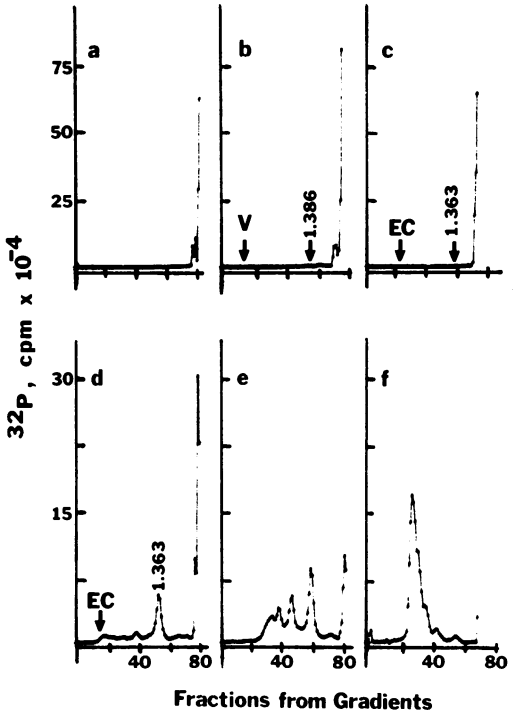

FIG. 1. *Conditions leading to stable products of association of adenovirus capsids and DNA in CsCl density gradients. Each reaction contained 1.2 μg of ^{32}P-labeled Ad3 DNA (1.3 × 10^6 cpm/μg) in 3.0 ml of 0.1 M Tris-hydrochloride, pH 8. After incubation with viral particles, the reactions were adjusted to 1.33 g/ml with CsCl and centrifuged overnight at 4°C in an SW56 rotor. The centrifuge tubes were punctured at the bottom, and ca. 80 single-drop fractions were collected. ^{32}P was measured as Cerenkov radiation in aqueous solution with a liquid scintillation counter. The arrows in (b, c, and d) indicate the centers of absorbance at 280 nm due to the empty particles or virions which accompanied the DNA during centrifugation. The numbers indicated in the same panels indicate the density, determined from refractive index, at which products of stable one-to-one, particle-to-DNA association were expected. The bottom of the gradients is at the right of each profile, and the last point shows the radioactivity deposited on the centrifuge tube itself. (a) Complete pelleting of the DNA from a reaction which contained no viral particles. (b) Reaction in which 1.5 × 10^{12} virions were incubated in 0.05 M CsCl with the viral DNA for 20 min at 37°C. (c) Distribution of radioactive DNA after incubation with 1.2 × 10^{12} empty particles of Ad3 in 2.6 M CsCl, the buoyant solution used in the isopycnic gradients. (d, e, and f) Gradients for reactions which contained 1.2 × 10^{12} empty Ad3 capsids together with the viral DNA in 0.05 M CsCl–0.1 M Tris-hydrochloride, pH 8. (d) Results obtained with incubation at 4°C for 20 min. (e and f) Distributions of products after incubation at 37°C for 3 and 20 min, respectively. EC, empty capsids; V, virions.*

higher than the original density interface to the bottom of the tube.

RESULTS

Stable products of empty capsid-DNA association in vitro. The affinity of empty adenovirus capsids for DNA in vitro was first observed by the appearance of stable association products in equilibrium CsCl density gradients. Centrifugation led to pelleting of Ad3 or Ad7 DNA (23 × 10^6 daltons), while empty capsids banded at their characteristic buoyant position, 1.30 g/ml. Interactions leading to a presumed one-to-one product of DNA and capsid association were predicted to have a buoyant density of about 1.35 g/ml, similar to the density of virions. Figure 1 shows the results typical of such experiments. ^{32}P-labeled viral DNA alone (Fig. 1a) was fully removed from the upper portion of the gradient and pelleted at the bottom of the tube. In low-salt solutions the interaction of empty capsids with viral DNA gave rise to products which appear in paucidisperse distributions of density classes (Fig. 1d, e, and f). A striking feature of these distributions is the shift to lighter density classes as the extent of particle-DNA interaction increases. The extent of the interaction and the shape of the distribution of products in the density gradient is influenced by the time of incubation, temperature, salt concentration, and the concentrations of the reactant empty capsids and DNA molecules. Figure 1e and f show the products resulting from incubation in 0.05 M CsCl at 37°C for 3 and 20 min, respectively. Figure 1d and f show matched incubations for 20 min at 4 and 37°C, respectively.

Empty capsids incubated with the DNA in 2.6 M CsCl before centrifugation showed no evidence of the anticipated association products (Fig. 1c). Virions incubated with DNA in solutions of lower salt concentrations (0.05 M CsCl) also showed no evidence of stable association (Fig. 1b), although such conditions were found to be favorable for formation and isolation of the products of empty capsid-DNA association (Fig. 1d, e and f). Highly purified Ad3 and Ad7 incomplete particles, containing a short fragment from the left end of the viral genome (24), also failed to stably bind to other DNA molecules in vitro (results not shown). Additional experiments of the same type have revealed no influence of magnesium (10 mM $MgCl_2$) or nucleotides (ATP or GTP, 10 mM) on the yield or distribution of the empty capsid-viral DNA association products. Addition of 0.5% Sarkosyl during or subsequent to the incubation disrupted the particles and permitted the DNA to pellet to the tube

bottom during the centrifugation (results not shown).

Shallower density gradients, centrifuged in angle rotors, were fractionated to determine the density of the several classes of association products (Fig. 2). Five discrete density classes representing association of Ad3 empty capsids with Ad3 viral DNA were observed, with an additional shoulder of material on the low-density side of class 5. These species were recovered in the density range of 1.31 to 1.37 g/ml. The distribution and the densities of the products suggest a stochastic type of interaction between the empty capsids and the DNA molecules in solution. The inset in Fig. 2 shows a computer-drawn simulation based on random binding of successive particles to individual DNA molecules, anticipating a Poisson distribution of products. Corrections were made for the expected increase in the extinction at 254 nm, due to increments of protein per DNA molecule, and decrease in band width for increased molecular weights. This and similar experiments have spanned a range from 0.1 particle per DNA to more than 10 particles per DNA; the distributions of association products in the gradients were consistent with the stochastic model throughout this range. Density gradients from reactions driven with excess DNA failed to show any diminution in the yield of one-to-one capsid-to-DNA product or evidence of species at higher densities suggestive of more than one DNA molecule per capsid. The empty capsids, therefore, are limited to interaction with one DNA molecule in vitro, under the conditions we have described for the stable association.

Radioactively labeled empty capsids (24) have also been used to follow the course of in vitro interaction with viral DNA. When excess amounts of DNA are used, the pool of empty particles is saturated; only about 16% of the total pool of capsids prove competent to interact with the viral DNA to make the one-to-one class of product. Under the conditions used in Fig. 2, complete reaction of this 16% competent empty capsid pool with the available DNA in the reaction represented an average of 3.6 particles per DNA molecule, a value consistent with the similarity of the observed distribution of products in the density gradient and the simulation based on the assumption of stochastic binding of an average of 3.6 particles per DNA. The residual empty capsid pool was recovered from reactions driven with saturating amounts of DNA. This residual pool no longer contained particles which could interact with additional viral DNA in subsequent incubations (results not shown). Thus, a limited fraction of purified

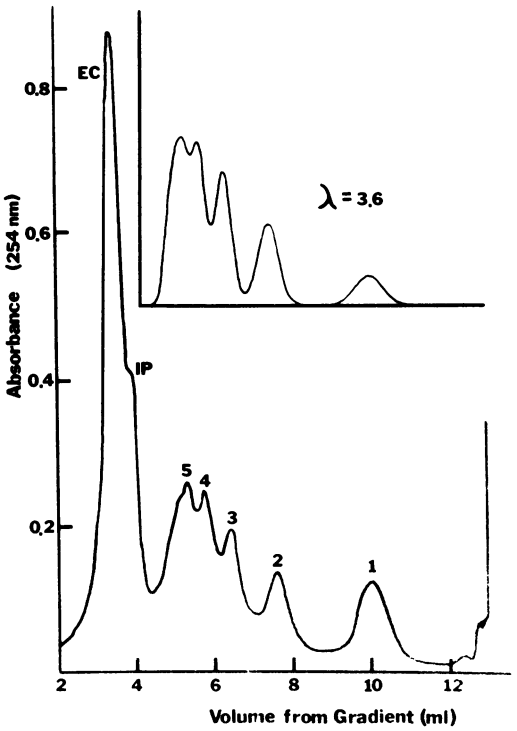

FIG. 2. *Distribution of Ad3 DNA-empty capsid association products suggest binding successive particles to the DNA molecules. A reaction mixture was prepared by incubation of 0.67 μg of Ad3 DNA per ml with 4×10^{11} empty capsids per ml for 30 min at 37°C. After addition of CsCl to 1.32 g/ml, the sample was centrifuged for 18 h at 40,000 rpm in a Ti75 rotor at 4°C. The resulting density gradient was fractionated by passage through a flow cell from the top of the gradient to a fraction collector, with the absorbance recorded at 254 nm. Refractive index determinations were made for density calculations immediately after collection of 60 fractions. The absorbance profile of the gradient shows the residual empty capsids (EC), a shoulder due to contaminating incomplete particles (IP), and at least five well-defined density classes of capsid-DNA association products. The inset shows a computer-simulated profile of reaction products. Positions corresponding to the band centers were calculated by the predicted densities of Ad3 DNA with integral numbers of 123-megadalton protein capsids (Fig. 3). The relative amounts of the products having different numbers of particles per DNA were estimated from a Poisson distribution having a mean value of 3.6. The band widths of the different classes of particles were estimated relative to the distribution of the one-to-one-class product at 1.363 g/ml, considering the band narrowing due to the increments in molecular weight on binding successive particles. The extinction of the capsid protein and DNA at 254 nm was also incorporated into the simulation to reflect the slightly increased sensitivity for detection of the higher-molecular-weight reaction products.*

empty capsids could interact with viral DNA in vitro and each capsid was limited to interaction with only one DNA molecule.

The densities of DNA-capsid association products were determined by using different homogeneous viral and phage DNA preparations (Fig. 3). The values determined for the buoyant densities were compared to 1.30 g/ml (100% protein in empty capsids) and 1.7 g/ml (for 100% DNA) to calculate the weight fractions of DNA in the various products of capsid-DNA association. Given these values and the molecular weights of the DNA used in each of the experiments, a consistent value of (123 ± 5) × 10^6 was obtained for the molecular weight of empty particles which bind to double-stranded DNA in vitro. This is significantly lower than the value of 156 × 10^6 ascribed to the protein moiety of adenovirions (17).

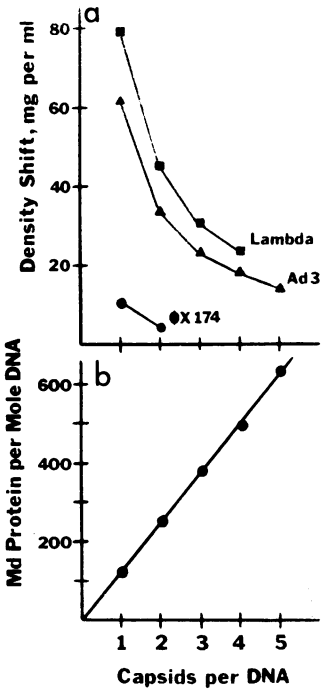

FIG. 3. *Density shifts for different numbers of empty capsids binding to different virus and phage DNA. Reaction products similar to that described in the legend to Fig. 2 were prepared, centrifuged, and fractionated by using Ad3 DNA (23 megadaltons), phage lambda DNA (31 megadaltons), or phage φX174 DNA (3.5 megadaltons). (a) Densities of the products representing different numbers of capsids bound per DNA, relative to the buoyant density of the empty capsids (1.30 g/ml). The density 1.7 g/ml, corresponding to 100% DNA, was used to calculate the percent DNA in each of the reaction products obtained with the different DNA species. These values, and the molecular weight of the respective DNA species used, permitted calculation of the molecular weight of the protein component of the association products. (b) Estimates obtained from the different DNA species used. The slope of the line corresponds to an empty capsid molecular weight of (123 ± 5) × 10^6.*

Kinetics and salt dependence of empty capsid-DNA association. In solutions with less than 0.1 M CsCl (or NaCl) the DNA-capsid association was very rapid. Typical reactions with 10^{11} capsids and 10^{11} DNA molecules per ml were complete within minutes at 37°C. Because the stable association products were not formed at all in 2.6 M CsCl, the time course could be followed by withdrawing portions in rapid succession from the low-salt reaction mixture into solutions ready for isopycnic centrifugation. The extent of the reaction was determined by the fraction of DNA floating up into the gradient after centrifugation. Figure 4a shows the time course of a typical reaction. Matched reactions were then incubated 30 s at 37°C at different salt concentrations before addition of the CsCl for centrifugation (Fig. 4b). The yield of DNA-capsid association products was markedly reduced as the salt concentration

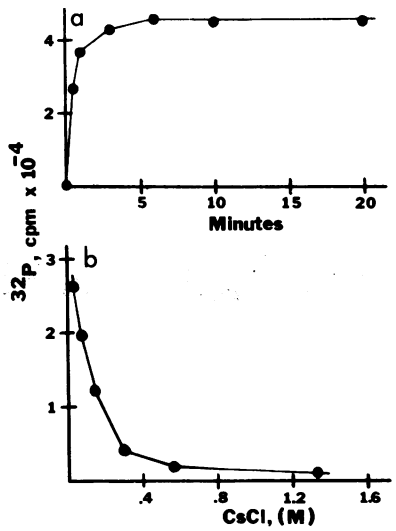

FIG. 4. *Kinetics and salt dependence of empty capsid-DNA association. (a) Time course of a reaction incubated at 37°C in 0.03 M CsCl–0.1 M Tris-hydrochloride (pH 8) with 3 × 10^{11} empty capsids per ml and 0.7 μg of ^{32}P-labeled Ad3 DNA (1.8 × 10^{10} DNA molecules per ml) per ml. Samples were withdrawn for analysis similar to the centrifugation experiments shown in Fig. 1d, e, and f. The extent of the reaction is determined by the radioactivity floating in association with buoyant particles in the density gradients. (b) The extent of reactions incubated under the same conditions described above for 30 s at 37°C, but at different concentrations of CsCl.*

of the reaction mixture was increased, yet the products formed at low salt were stable in high-salt solution. The reaction was not irreversible, as rebanding of isolated complexes in successive density gradients led to decreased yields of the complex; breakdown products were recovered as buoyant empty particles and pelleted DNA. The dissociation of particles from DNA has not been carefully analyzed. The rate of dissociation during storage of the purified complexes was less than 10% per week at 4°C.

Random binding sites on DNA molecules. The apparently random nature of the capsid-DNA association was more thoroughly examined by analysis of competitive association of restriction fragments of viral DNA with limiting quantities of empty capsids. The experiment shown in Fig. 5 is based on competitive association of empty capsids with Ad3 DNA cleaved by endo R·*Xho*I. A mixture of empty Ad3 capsids plus *Xho*I-cleaved, ^{32}P-labeled Ad3 DNA was incubated, and portions were withdrawn for step gradient centrifugation as described in Materials and Methods. The particle-associated DNA was recovered, dialyzed, and analyzed by gel electrophoresis to determine the relative yield of the different restriction fragments. The eventual extent of association, about 60% of the mass of the DNA, suggests a preferential interaction of the empty capsids with the larger DNA fragments of the mixture. This suggestion is further supported by the distribution of recovered fragments shown in Fig. 4b. The control sample of unfractionated DNA shows the distribution of radioactivity expected for the case of equimolar quantities of the *Xho*I fragments of DNA. The capsid-associated DNA fragments show a length-weighted bias in the recovery. This is expected if the rate of association of capsids with DNA molecules is proportional to the length of the DNA (number of binding sites per molecule). This is furthermore consistent with the earlier suggestion of binding to randomly distributed sites along the contour of double-stranded DNA.

Similar results were obtained when one-to-one capsid-to-DNA complexes were purified, treated with restriction enzymes, and recentrifuged in CsCl step gradients as described above. DNA fragments remaining in association with the capsids were again recovered in length-weighted stoichiometry (results not shown).

Nuclease sensitivity of DNA in the empty capsid-DNA association products. Samples of one-to-one capsid-to-DNA complexes were prepared and purified by density gradient centrifugation after incubation of empty capsids with ^{32}P-labeled Ad3 DNA. After dialysis against 0.10 M Tris-hydrochloride (pH 8), the samples were treated with DNase I, endo R·*Bam*HI, and endo R·*Hin*dIII in parallel with samples containing equal quantities of radioactive DNA alone. Electrophoresis in agarose gels (Fig. 6) revealed no difference in nuclease susceptibility of viral DNA free in solution or associated with empty adenovirus capsids. Significant lengths of DNA do not appear to be protected within the confines of the bound particles.

Electron microscopy of empty capsids bound to DNA. Empty capsids and capsids bound to DNA molecules were examined by electron microscopy by using a brief period of glutaraldehyde fixation in conjunction with uranyl acetate negative staining (Fig. 7). No apparent difference could be found between the subpopulation of capsids which bound to Ad3 viral DNA and the residual, nonbinding empty capsid pool. The size and structure of the empty parti-

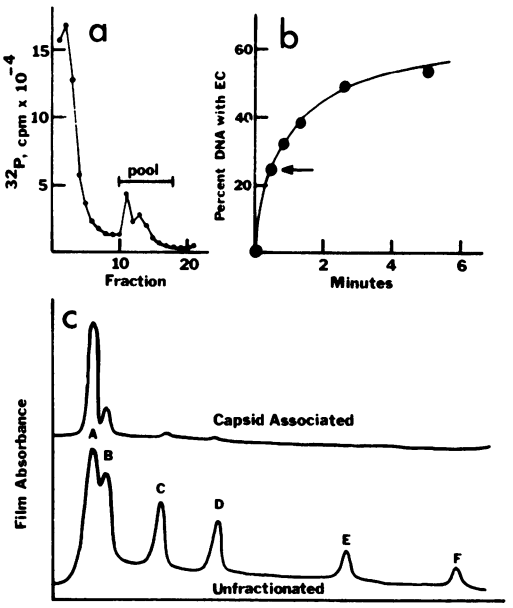

FIG. 5. *Evidence for randomly distributed capsid-binding sites on DNA based on the relative rates of restriction fragment associations with empty capsids. A reaction mixture was prepared with 6 × 10^{11} empty Ad3 particles per ml and 2.6 μg of Xho-I cleaved, ^{32}P-labeled Ad3 DNA (5 × 10^{11} DNA molecules per ml) per ml was incubated at 37°C. Samples were withdrawn into 1.5-g CsCl/ml for step gradient centrifugation as described in the text. (a) Profile of radioactivity resulting from fractionation of the first sample, withdrawn after 22 s at 37°C (the tube bottom is fraction 1). (b) Continued time course of the reaction. (c) Radioactive fractions floating in association with the buoyant capsids were pooled as indicated and dialyzed before agarose gel electrophoresis. The lower trace shows the expected distribution of radioactivity in an equimolar collection of the XhoI cleavage fragments (fragment G, 1.1%, is not shown on the gel). EC, empty capsids.*

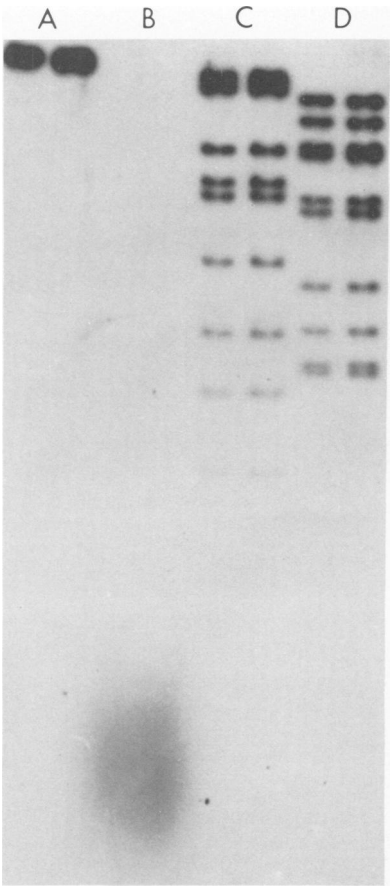

FIG. 6. *Nuclease sensitivity of the DNA in DNA-capsid association products.* ^{32}*P-labeled Ad3 DNA (2 × 10^6 cpm/μg) was used to prepare association complexes with Ad3 empty capsids under conditions similar to those described in the legend to Fig. 2. The resulting one-to-one class of association product was recovered from the gradients and dialyzed against 0.1 M Tris-hydrochloride (pH 8) and then adjusted to 0.01 M $MgCl_2$ and 0.05 M NaCl. Equal quantities (2 × 10^5 cpm) of naked Ad3 DNA and DNA associated with single capsids were treated as described below, adjusted to 0.02 M EDTA, and subjected to electrophoresis in a 1% agarose slab gel. The left track of each pair shows the naked DNA sample, and the right track shows the treated capsid-DNA association product. (A) The intact viral DNA recovered from each sample. (B) The radioactivity remaining after treatment of the samples for 30 min at 37°C with 10 μg of DNase I per ml. (C and D) The samples after restriction with endo R·BamHI and endo R·HindIII, respectively. These results indicate little difference in the accessibility of the DNA associated with single capsids and naked DNA for the actions of the endonucleases used. Control experiments (not shown) revealed that the incubation conditions (30 min, 37°C, solutions as described but without addition of enzymes) resulted in less than 25% breakdown of the association complexes into empty capsid and free DNA components.*

cles in each case were consistent with the reports on empty particles and incomplete adenovirus particles published by others (21). Threadlike masses of collapsed DNA are seen in the micrographs of negatively stained DNA-capsid complexes. The brief period of fixation rendered the particles sufficiently stable to survive the conditions of spreading DNA in protein films as described in Materials and Methods. This modification of the Kleinschmidt technique provides the clearest illustration of the relationship of the bound adenovirus capsids to the contour of the linear DNA molecules (Fig. 8). Length measurements of DNA with and without bound particles show that little more than the length of DNA physically obscured by the bound particle, about 0.1 μm, could be within the particle.

Mixture of the association products with the cytochrome *c* for film spreading led to displacement of most (>95%) of the particles originally bound to the DNA. Of the remaining DNA-bound capsids, there appeared to be a disproportionate fraction, about 15 to 20%, located at one end of the Ad3 viral DNA. This does not agree with the randomly distributed sites of bound particles suggested by the distribution of association products or the recovery of different particle-associated restriction fragments (Fig. 2 and 5).

Polypeptides of empty capsids and capsid-DNA association products. Conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to analyze viral polypeptides of purified Ad3 virions, empty capsids, and the products of empty capsid-DNA association (9; Tibbetts, Green, and Silvernail, manuscript in preparation). No major differences are observed in comparison of the polypeptide compositions of those empty capsids which can or cannot bind to the viral DNA. The relative amounts of the capsid shell polypeptides (hexon, penton, fiber, 27 K pVI and IX) are similar to those observed with the corresponding peptides of mature Ad3 virions. Core polypeptides V and VII (or pVII) are notably missing from the empty particles and from the subpopulation of empty capsids which is competent to stably associate with double-stranded linear DNA in vitro.

DISCUSSION

The substantial evidence implicating empty viral capsids as intermediates in the adenovirus assembly pathway (17) led to our exploration of the potential for using these particles to study events related to DNA packaging in vitro. This report describes our investigation of the affinity of purified empty adenovirus capsids for double-stranded DNA. We have utilized the empty cap-

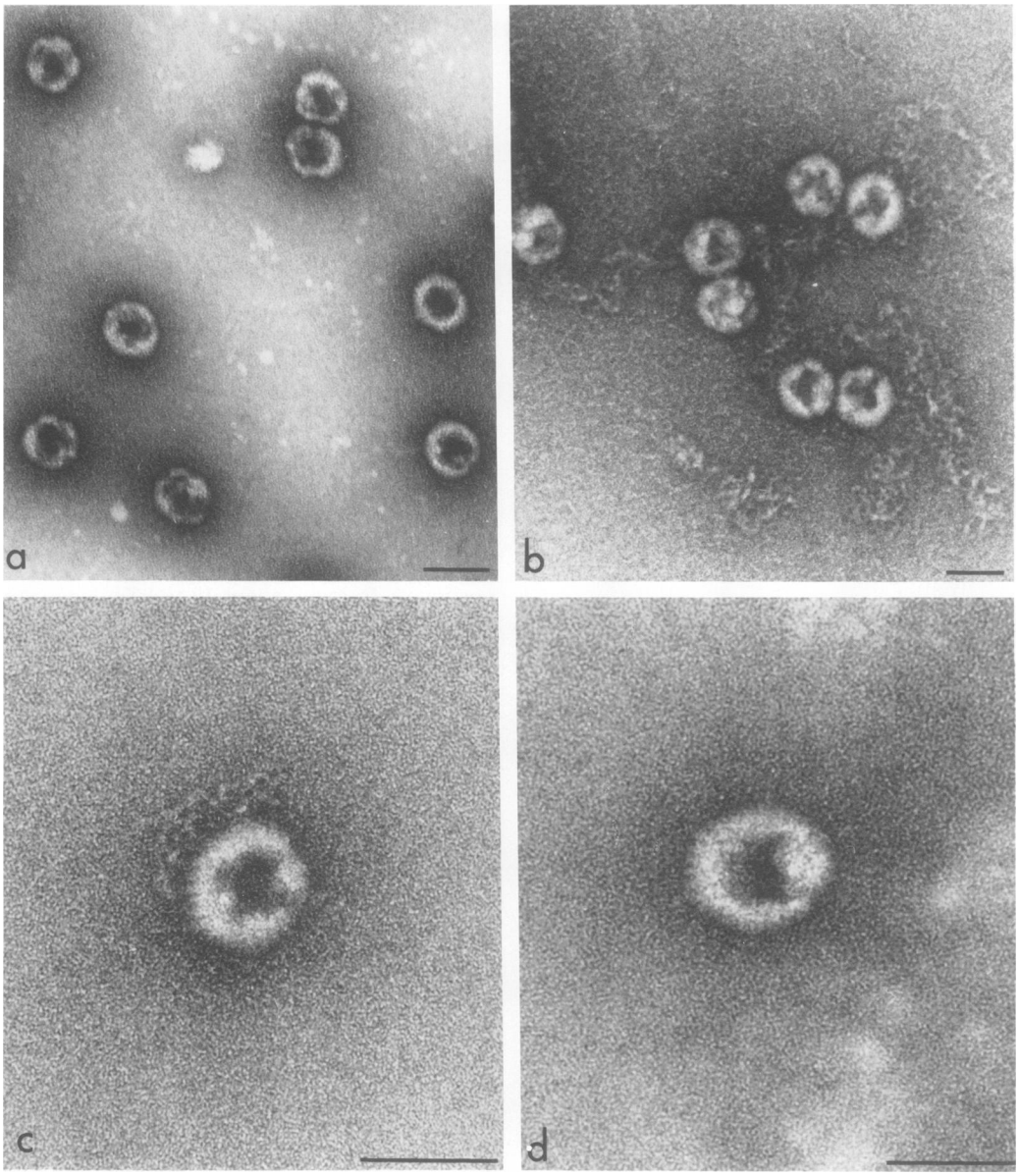

FIG. 7. *Electron micrographs of negatively stained empty capsids and capsids associated with DNA molecules in vitro. (a) Particles from the residual empty capsid pool after those particles competent to stably interact with the viral DNA have been removed. (b, c, and d) (higher magnification) Particles of the one-to-one class of capsid-DNA association. Threadlike accumulations of collapsed DNA can be seen in the periphery of the negatively stained capsid-DNA complexes. Holes on the surface of the empty particles are apparent in each of the preparations. The particles appear largely intact, but are somewhat prone to collapse under the dehydration conditions used in preparation of the grids. The shape and size of the particles (about 70 to 80 nm) are consistent with the shell structure of the adenovirus. Bars, 100 nm each.*

sids of Ad3 and Ad7 because these particles are characteristically abundant and readily purified from cells infected by subgroup B human adenoviruses. We have not yet determined whether similar particles from other adenovirus subgroups share the same capacity for stable association with DNA in vitro.

The empty adenovirus capsids rapidly associated with double-stranded DNA in low-salt solution, below 0.1 M CsCl or NaCl. The products

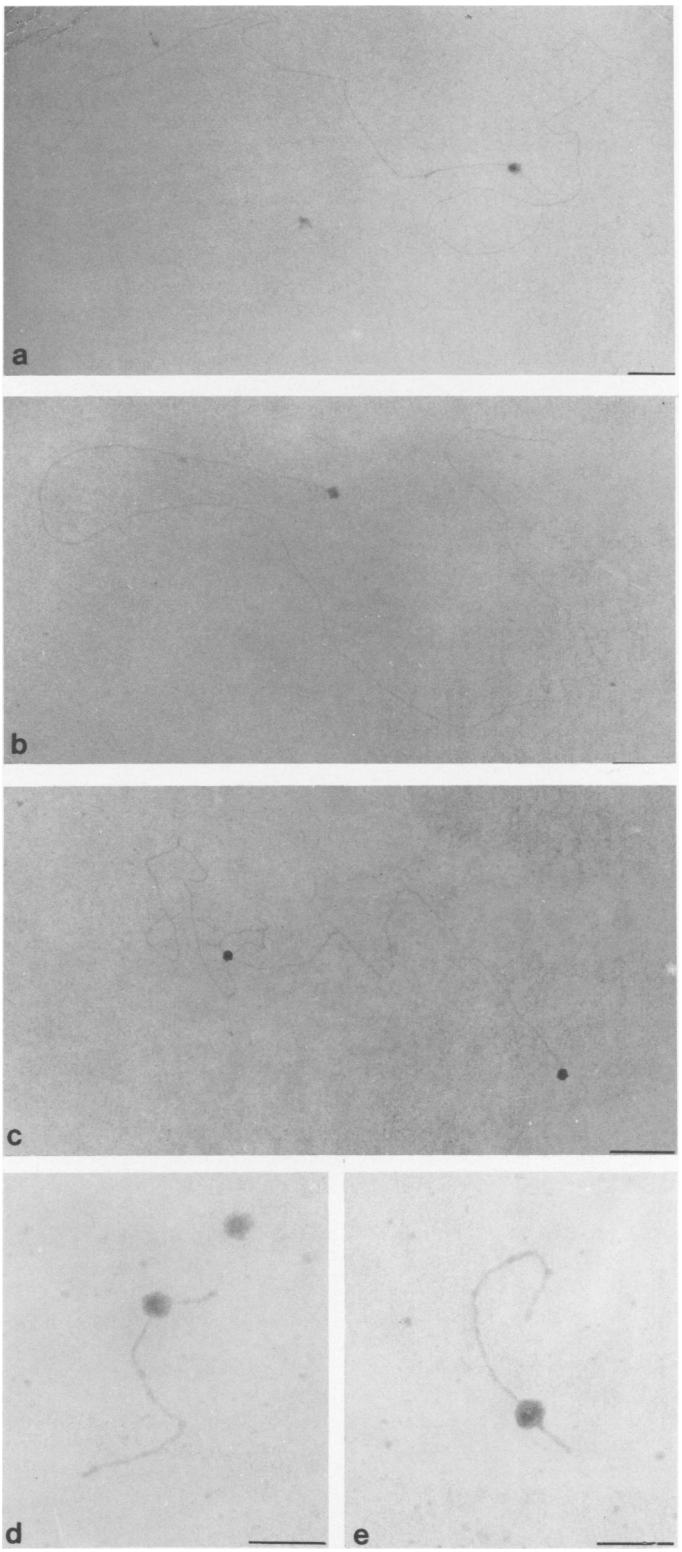

FIG. 8. *Electron microscopy of capsid-DNA association products in cytochrome c films. Samples of capsid-DNA association products were spread in protein films, adsorbed to grids, and stained as described in the text. (a, b, and c) Full-length Ad3 viral DNA molecules with bound Ad3 empty capsids. About 10 to 20% of residually bound particles are found at the end of the viral DNA molecules. (d and e) Ad3 empty capsids bound to linear, XhoI-cleaved φX174 DNA molecules. The hexagonal outline of the particles, consistent with icosahedral shell structure, is readily apparent. (a, b, and c) Bars, 500 nm; (d and e) bars, 250 nm.*

of association, once formed, were stable in the high-salt conditions of CsCl density gradients. The inhibition of the formation of stable complexes with increasing salt concentrations is reminiscent of the ionic interaction of DNA with purified fiber antigen described by Levine and Ginsberg (15). Fiber-DNA binding may in fact account for the rapidity of the association process, bringing particles and DNA into sufficient proximity for a substantially stronger, perhaps in a cooperative capsid protein-DNA interaction. Virions and incomplete particles do not remain stably associated with DNA at high salt concentrations, yet may be similar to the empty particles in their ability to interact with DNA at low salt. Centrifugation experiments in low-salt sucrose gradients or chromatography of viral particles on DNA bound to a fixed matrix may clarify distinct stages of capsid-DNA association in vitro.

Electron microscopy of negatively stained particles and the gross polypeptide composition of the empty particles suggest only a subtle structural difference distinguishing the particles which can or cannot form stable complexes with viral DNA in vitro. The capsids which bind to DNA in vitro have a molecular weight of 123×10^6, based on density shift measurements (Fig. 3). This is consistent with a nearly intact icosahedral shell of the capsids, missing little more than a single pentagonal vertex or one of the 20 triangular facets. Single discontinuities in empty capsids of Ad3 were reported earlier by Sundquist et al. (21) and suggested to be the site for entry of a DNA-core complex. The affinity of empty capsids for double-stranded DNA may thus result from the organization of major polypeptides, exposing a DNA binding surface within the shell. On the other hand, we have not investigated less abundant polypeptides, perhaps only one per capsid, which might play the determining role in the capsid-DNA association process.

Aposhian et al. (1) reported the ability of purified empty particles of polyoma virus to bind to superhelical molecules of the viral DNA in vitro. The products of capsid-DNA association were not stable in high-salt solution and were not characterized with regard to the specificity of the DNA in the reaction. The failure of purified polyoma virions to bind to viral DNA under the conditions suited for the empty capsid association led the authors to similar considerations of the nature of the interaction as we have made for the case of association of empty adenovirus capsids with double-stranded DNA. Their results and ours may reflect a general property of empty capsid structures as intermediates in the assembly of DNA-containing animal viruses.

Thermodynamic considerations (19) suggest that electrostatic repulsion within the acidic DNA itself is the major positive free-energy barrier to packaging the viral DNA genome. Mechanical and entropic barriers should be very limited in comparison. Given charge neutralization by interaction of the DNA with basic core polypeptides, however, these other considerations may determine the actual energy barrier for DNA packaging in vitro. It certainly seems likely that addition of polypeptides V and VI (or pVII) will be necessary to achieve significant enclosure of the DNA within the capsids bound to DNA in vitro. These proteins are readily purified from infected cells by acidic urea extraction followed by phosphocellulose chromatography (22; Giam, unpublished data with Ad3). The same methods which we have used to characterize the products of in vitro capsid-DNA association can be used to assess the extent of DNA engulfment within the bound particles after incubation with the basic core proteins.

An earlier report by Winters and Russell (28) suggested that assembly of infectious adenovirus may be achieved in extracts of cells infected by the virus but blocked in assembly under conditions of arginine deprivation. Fractionated components from productively infected cells are required to promote the assembly of virus. We are now conducting experiments in which purified Ad3 empty capsids and the empty capsid-DNA association products described in this paper are added to arginine-deprived, Ad3-infected cell extracts. Prelabeled cells and empty capsid-DNA association products permit identification of the sources of specific proteins or DNA in products that result from this type of mixing experiment. We have already observed that purified empty capsids associate with fragments of viral DNA when added to sonic extracts of ^{32}P-labeled, arginine-deprived, Ad3-infected cells (Yuan and Tibbetts, unpublished data).

There appears to be a left-end-specific polarity of empty capsid association with the viral genome during packaging of the DNA in vivo (24). The mechanism underlying this phenomenon is particularly intriguing because the ends of the linear adenovirus genome are physically identical (13, 29). The lengths of inverted terminal repetitions in adenovirus DNA, determined from base sequence analysis (20), suggest that a site which could determine the left-end polar entry of DNA into empty capsids must be located at least 100 to 150 base pairs from the end of the molecule. Under the conditions of formation of stable association complexes in vitro, there is no apparent preference for any sequence of DNA. Yet very little DNA, if any, appears to be collapsed within bound particles

in a manner that would restrict motion of the particles along the DNA molecule, a process of diffusion in one dimension. Eventual recognition of an interaction with the putative specific binding site may be possible for the empty particles as presently purified, or other factors may exist which are not yet included in our in vitro system.

ACKNOWLEDGMENTS

We thank Ellen Silvernail for her supportive contributions and excellent technical assistance through the course of this work. Kathryn Woodle-Book worked in a painstaking, deliberate, and thoughtful manner, successfully adapting methods to new materials, to obtain the electron micrographs of the capsid-DNA complexes.

This investigation was supported by Public Health Service grant CA-17130 from the National Cancer Institute.

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