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Adenovirus L1 52- and 55-Kilodalton Proteins Are Present within Assembling Virions and Colocalize with Nuclear Structures Distinct from Replication Centers

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Analysis of a temperature-sensitive mutant, Ad5*ts*369, had indicated that the adenovirus L1 52- and 55-kDa proteins (52/55-kDa proteins) are required for the assembly of infectious virions. By using monoclonal antibodies directed against bacterially produced L1 52-kDa protein, the L1 52/55-kDa proteins were found to be differentially phosphorylated forms of a single 48-kDa polypeptide. Both phosphoforms were shown to be present within all suspected virus assembly intermediates (empty capsids, 50 to 100 molecules; young virions, 1 to 2 molecules) but not within mature virions. The mobilities of these proteins in polyacrylamide gels were affected by reducing agents, indicating that the 52/55-kDa proteins may exist as homodimers within the cell and within assembling particles. Immunofluorescence analysis revealed that the 52/55-kDa proteins localize to regions within the infected nucleus that are distinct from viral DNA replication centers, indicating that replication and assembly of viral components likely occur in separate nuclear compartments. Immunoelectron microscopic studies determined that the 52/55-kDa proteins are found in close association with structures that appear to contain assembling virions. These results are consistent with an active but transient role for the L1 products in assembly of the adenovirus particle, perhaps as scaffolding proteins.

A series of incomplete viral particles in cells infected with wild-type and mutant adenoviruses have been identified, and they can be ordered into a logical series of putative assembly intermediates (for a review, see reference 22). Substantial quantities of empty capsids accumulate in cells infected with wild-type adenoviruses. Pulse-chase experiments have led to the proposal that they are then partially filled to form intermediate particles, which are composed of capsids associated with the left end of the viral chromosome. Intermediate particles are found in small quantities within cells infected with wild-type group C adenoviruses (e.g., adenovirus type 2 [Ad2] and Ad5). Ad2 and Ad5 mutants that accumulate these particles have been described, and wild-type group B adenoviruses (e.g., Ad3 and Ad7) generate them in large quantities. Complete incorporation of a core (a viral chromosome packaged in virus-coded basic proteins) into an empty capsid generates a so-called young virion. The young virion is matured by the action of a virus-coded proteinase to form a mature, infectious virion. One interpretation of these intermediates, then, suggests that cores associate with empty capsids, initially forming intermediate particles and ultimately producing young virions as the encapsidation process is completed (22). The process of virion assembly requires numerous virus-coded proteins, including proteins not found within the mature virion.

The adenovirus L1 region encodes several proteins important for assembly of infectious virus particles, the 52- and 55-kDa proteins (52/55-kDa proteins) and the IIIa protein. Protein IIIa is a structural component of the virion (3). The 52/55-kDa proteins are nuclear phosphoproteins that are highly related in amino acid sequence (16), but they have not been detected in virions (12, 15, 16). Mutations in either the 52/55-kDa proteins (12) or IIIa protein (5, 7) lead to accumulation of intermediate particles. The 52/55-kDa mutant, Ad5*ts*369 (12), represents a unique class of assembly mutant; its altered proteins appear to be involved in the encapsidation step of virion assembly, but the proteins themselves are not components of mature virions.

To further investigate the function of the L1 52/55-kDa proteins, we generated a series of monoclonal antibodies directed against bacterially produced protein. Using these antibodies, we have determined that the two proteins are produced as a result of the differential phosphorylation of a 48-kDa precursor protein. The 52/55-kDa protein species are located within all known virus assembly structures, which is consistent with a role in virus assembly. They are found within empty capsids, assembly intermediates, and young virions but not within mature virions. Immunofluorescence analysis demonstrated that the 52/55-kDa proteins are located at sites within infected nuclei that are distinct from viral replication centers, suggesting that replication and encapsidation take place at separate locations within the infected nucleus. Immunogold labeling experiments localized the proteins to the vicinity of putative virion assembly intermediates.

MATERIALS AND METHODS

Cell culture and viruses. HeLa and 293 (10) cells were propagated as monolayer cultures in Dulbecco's minimal medium supplemented with 5 to 10% calf serum. For electron microscopic analysis of extracted cells, HeLa cells were propagated in monolayer cultures on Mylar film (21) in medium supplemented with 5% calf serum.

Ad5*wt*300 is the parental, wild-type adenovirus (14). Ad5*ts*369 is a temperature-sensitive mutant containing a two-amino-acid substitution in the L1 open reading frame that encodes the 52/55-kDa proteins (12). Ad5*pm*382 is an

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Ad5 variant which lacks the i-leader splice acceptor site (28). Because of this alteration in splicing, the virus overexpresses the L1 52/55-kDa proteins. Ad2*ts*1 is a temperature-sensitive mutant defective in the function of the L3 23-kDa endoproteinase at the nonpermissive temperature (32, 33). This virus was provided by S. J. Flint (Princeton University). Ad5*wt*300 and Ad5*pm*382 were propagated in HeLa cells at 37°C, while Ad2*ts*1 and Ad5*ts*369 were grown in HeLa cells at 32°C. Viruses in cell lysates were titrated by plaque assay on 293 cells at either 32 or 37°C.

Antibodies. To generate monoclonal antibodies to the adenovirus L1 52/55-kDa proteins, a plasmid construct, pTRB1-68, was created; the construct contained the bacterial β-galactosidase protein encoded by pTRB1 (provided by E. Montalvo, Princeton University) fused to the amino terminus of the L1 52-kDa protein isolated from pA5-SalC (29). To prepare fusion protein, overnight cultures of bacterial strain Jm109 transformed with pTRB1-68 were diluted 1:20 in medium containing 50 mg of ampicillin per ml. After a 2-h incubation at 37°C, transcription of the β-galactosidase fusion gene was induced by the addition of 2.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). After an additional 2 h at 37°C, the bacteria were lysed by boiling for 7 min in buffer containing 2.5% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 10% glycerol, and 0.05% bromophenyl blue. Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (7% acrylamide gel [8]). The induced band of fusion protein was visualized by incubation of the gel in ice-cold 0.1 M KCl for 10 min. The region of the gel containing the induced band was excised, and the gel slice was equilibrated in three changes of buffer containing 25 mM Tris-HCl (pH 8.8), 190 mM glycine, and 0.1% SDS (8) and was placed in a dialysis bag. The protein was electroeluted from the gel slice for 16 h at 200 V at 4°C, released from the dialysis tubing by back elution for 2 min at 200 V, precipitated three times with ethanol, and stored in phosphate-buffered saline (PBS) at −20°C.

The fusion protein was employed as an immunogen to generate monoclonal antibodies. Briefly, 8- to 12-week-old BALB/c female mice were injected intraperitoneally with approximately 50 μg of antigen in RIBI adjuvant (RIBI Immunochemical Research Corp., Hamilton, Mont.). This was repeated after 2 weeks, and, with twice the amount of antigen, after an additional 3 weeks. Six days later, the mice were bled, and each animal's response to the antigen was initially determined by reacting serum with partially purified 52/55-kDa proteins spotted on nitrocellulose in a dot blot assay. One immunoresponsive mouse was given a booster injection, and its spleen cells were fused to SP2/0 myeloma cells. Approximately 750 hybridoma culture supernatants were screened by using the dot blot assay. Positive clones were expanded and rescreened by immunoprecipitation and protein blot assay. Six different hybridoma lines were obtained (Table 1).

The monoclonal antibody directed against the adenovirus E2 72-kDa DNA-binding protein (clone B6-8; gift of A. J. Levine, Princeton University) was described by Reich et al. (24). The anti-Ad2 rabbit polyclonal serum was a gift from S. J. Flint (Princeton University).

Labeling of 52/55-kDa proteins for immunoprecipitations. For phosphate labeling experiments, about 10^7 HeLa cells were infected at a multiplicity of 25 PFU per cell. At 20 h postinfection, the medium was removed and the cells were washed with warm PBS. The cells were labeled at 39.5°C for 4 h with 1.3 mCi of P_i ($H_3{}^{32}PO_4$, 285 Ci/mg; ICN Radiochemicals, Irvine, Calif.) in 2 ml of phosphate-free medium containing 5% calf serum. Whole cell lysates were prepared by resuspending the cells in 0.6 ml of TENN buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride. The lysate was then vortexed for 10 s and sonicated for 10 s at 50% output in a water-jacketed cup horn. The sample was then spun at 2,700 × *g* for 5 min, and the supernatant was frozen at −70°C.

Analysis of assembly structures. Particles were prepared (12) from cells infected with Ad5*wt*300, Ad5*ts*369, Ad2*ts*1, or wild-type Ad2 and further purified by equilibrium density centrifugation in CsCl (median densities, 1.34 g/cm^3 for virions and young virions, 1.31 g/cm^3 for intermediates, and 1.29 g/cm^3 for empty capsids). In some cases, a further purification was undertaken in which the virion particles were mixed with an equal volume of 1.65-g/cm^3 CsCl and underlaid beneath a preformed 1.2- to 1.4-g/cm^3 CsCl gradient before centrifugation at 62,500 × *g* for 3 h.

For analysis of proteins in assembly structures, samples were concentrated by precipitation by addition of 3 volumes of TNE (10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 7.5) and 5 volumes of acetone. Samples were placed at −70°C overnight and then spun at top speed in a microcentrifuge at 4°C for 10 to 30 min, dried in a rotary evaporator, and resuspended in electrophoresis sample buffer. Electrophoresis and autoradiography were performed as described previously (12).

Immunoprecipitations. For immunoprecipitation of proteins from purified young virions, the virions were incubated for 5 min in the presence or absence of 4 M guanidinium hydrochloride and then dialyzed into PBS before further use. Each sample was precleared (30 min at 4°C) by the addition of a one-fifth volume of *Staphylococcus aureus* cells (Pansorbin; Calbiochem Corp., San Diego, Calif.). After preabsorption, the cells were pelleted by centrifugation for 5 min in a microcentrifuge at 4°C and the supernatant was removed to a fresh tube. Hybridoma supernatant (100 μl) was then added to the lysate and allowed to incubate for 1 h at 4°C. Rabbit anti-mouse immunoglobulin G (IgG) (5 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, Pa.) (0.5 μl) was added to ensure recognition of antibodies by protein A. Finally, 3 mg of protein A-Sepharose beads (reconstituted in TENN) was added and allowed to mix for 1 h at 4°C. The beads were pelleted by centrifugation for 4 min in the microcentrifuge at 4°C and washed three times with 0.5 ml of SNNTE (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA) and once with 0.5 ml of NTE (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA). Pellets were then resuspended in electrophoresis buffer.

For samples to be treated with potato acid phosphatase, the beads were washed with 0.5 ml of 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.0, after being washed with SNNTE and NTE. The pellet was recovered in 50 ml of PIPES solution, and to this 0.5 U of potato acid phosphatase (grade I; Boehringer Mannheim Biochemicals) was added. The sample was incubated at 37°C for 30 min and then washed twice with 0.5 ml of 50 mM PIPES, pH 6.0, before being resuspended in electrophoresis buffer.

Indirect immunofluorescence. HeLa cell monolayers were grown to about 50% confluence and infected at a multiplicity of 25 PFU per cell at 39.5°C. Twenty-four hours later, the cells were prepared for indirect immunofluorescence as described by Ornelles and Shenk (21). In the case of double antibody labeling experiments, the primary antibody com-

TABLE 1. Characteristics of 52/55-kDa-protein-specific monoclonal antibodies

No.	Isotype[a]	Immunoprecipitation[b]	Western blot[b]	Immunofluorescence[c]
12	Unknown	(+)	(−)	ND
14	IgG2a	(+)	(+)	++
15	IgM	(+)	(+)	ND
16	IgG1	(+)	(+)	++
30	IgG2b	(+)	(+)	+
35	IgG2a	(+)	(+)	+

[a] All isotypes have kappa light chains. Unknown, not IgA, IgD, IgG, or IgM isotype.
[b] (+), Active in the assay; (−), inactive in the assay.
[c] ND, not determined; ++, exceptional staining observed by immunofluorescence; +, good staining observed by immunofluorescence.

plexes were fixed with PBS containing 1 mM Mg^{2+} (PBS-Mg^{2+}) plus 4% paraformaldehyde for 5 min at room temperature. After fixation, the samples were washed five times with PBS-Mg^{2+} containing 0.5% bovine serum albumin (BSA) and incubated for 30 min with 1 ml of goat anti-mouse immunoglobulin G (IgG) (1:1,000 dilution in PBS-Mg^{2+} plus 0.5% BSA). This treatment blocked any unbound antibody from further reaction with anti-mouse antibodies. Samples were fixed with PBS-Mg^{2+} plus 4% paraformaldehyde as described above before addition of the second antibody. For the double labeling experiment, the first antibody used was specific for the 52/55-kDa proteins (Table 1, no. 16) and recognized by fluorescein-conjugated anti-mouse antibody (Jackson ImmunoResearch). The second antibody used was specific for the 72-kDa DNA binding protein (B6-8) and recognized by rhodamine-conjugated anti-mouse antibody (Jackson ImmunoResearch). Control experiments indicated that the fixation and blocking techniques described above effectively prevented recognition of the 52/55-kDa antibody (applied first) by the rhodamine-conjugated anti-mouse antibody (applied last). The DNA-binding dye DAPI (diaminophenyl indole; Polysciences, Inc., Warrington, Pa.) was included in the final wash at a concentration of 0.5 μg/ml.

Immunogold labeling and electron microscopic visualization of extracted cells. HeLa cells were infected at a multiplicity of 25 PFU per cell and processed 24 h later. Indirect immunogold labeling of extracted cells and staining and electron microscopic visualization of samples were as described by Ornelles and Shenk (21).

RESULTS

Monoclonal antibodies directed against bacterially produced L1 52-kDa protein. Since it was critical to be able to specifically assay for the L1 52/55-kDa proteins in assembly intermediates and to localize the proteins within infected cells, a set of monoclonal antibodies was produced. A construct in which the open reading frame encoding the 52/55-kDa proteins was fused to the 3′ end of the *lacZ* gene was prepared. By using this construct, pTRB1-68, an L1 52-kDa-protein–β-galactosidase fusion protein was produced in *Escherichia coli* and used to immunize mice. Six different monoclonal antibodies were produced (Table 1), all of which recognize epitopes within the N-terminal half of the viral protein (data not shown).

The 52- and 55-kDa proteins are phosphoforms of a 48-kDa polypeptide. In vitro translation of L1 mRNA generated both a 52- and a 55-kDa protein (1, 18), and analysis of tryptic peptides showed little difference between the two polypeptides (1, 16). It had been observed that the two proteins were phosphoproteins (16), so a labeling experiment was undertaken to determine whether the 52/55-kDa proteins are simply different phosphoforms of the same polypeptide. At 20 h after infection with wild-type virus, cells were labeled for 4 h by incubation in phosphate-free medium containing 1.3 mCi of $^{32}P_i$ per ml. The labeled 52/55-kDa proteins were immunoprecipitated from the extract in duplicate, and one of each pair of samples was treated with potato acid phosphatase to remove O-linked phosphates. The immunoprecipitates were probed by protein blot analysis by using a mixture of antibodies to 52/55-kDa proteins (Fig. 1A). A control precipitation lacking specific antibodies was included to monitor any reaction of the antibodies used in the protein blot analysis to the protein A-Sepharose beads used in immunoprecipitations. The 52/55-kDa proteins were converted to a single 48-kDa species when treated with phosphatase (Fig. 1A). When this protein blot was exposed to film (Fig. 1B), only the phosphorylated 52/55-kDa species were found to contain radioactivity. The 48-kDa species contained no detectable radioactivity, indicating that the phosphatase had efficiently removed all phosphate moieties.

From these studies, we conclude that the 52/55-kDa proteins are phosphoforms of a single polypeptide with an

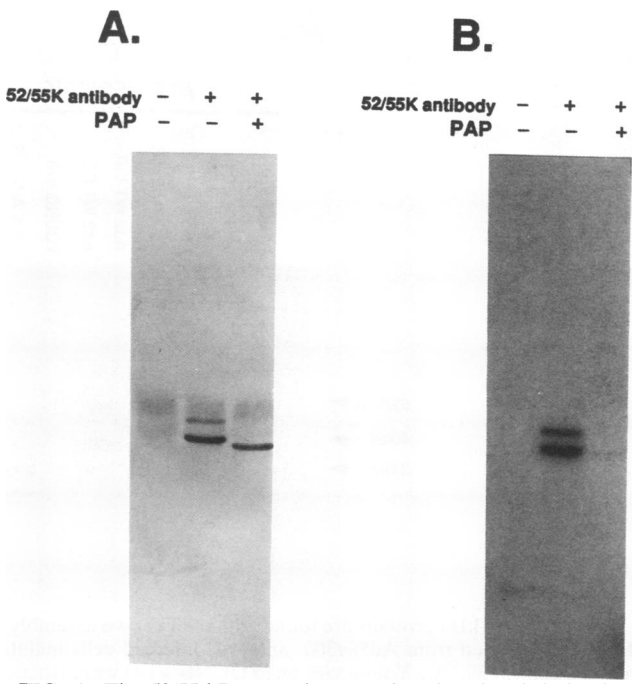

FIG. 1. The 52/55-kDa proteins are phosphorylated derivatives of a 48-kDa precursor protein. HeLa cells were infected with Ad5*wt*300 at a multiplicity of 25 PFU per cell and labeled for 4 h with $^{32}P_i$ beginning at 20 h after infection. Then the cells were harvested and the labeled proteins were immunoprecipitated with a pool of monoclonal antibodies specific for the 52/55-kDa proteins. Some immunoprecipitated samples were treated for 30 min at 37°C with 0.5 U of potato acid phosphatase (PAP) in 50 mM PIPES, pH 6. (A) Immunoprecipitates were probed by Western blot (immunoblot) analysis with a mixture of monoclonal antibodies specific for the 52/55-kDa proteins (no. 14, 15, 16, and 30). The proteins were visualized by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG plus IgM, and color was developed by addition of a cleavable chromophore. (B) Western blot shown in panel A, exposed to X-ray film.

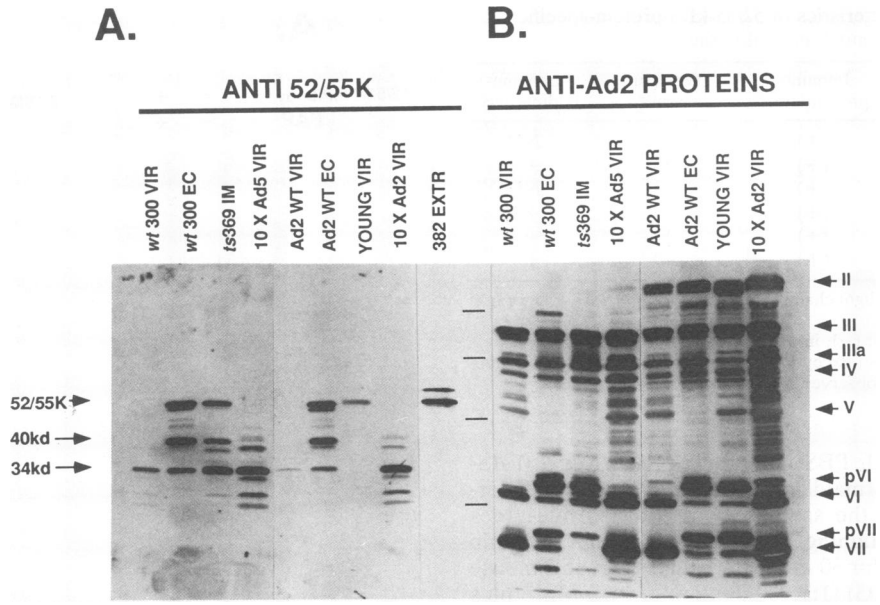

FIG. 2. 52/55-kDa proteins are found within all known assembly structures but not within mature virions. Virions (VIR) and empty capsids (EC) were isolated from Ad5*wt*300- and Ad2-infected cells maintained at 37°C. Intermediate particles (IM) were isolated from Ad5*ts*369-infected cells at 39.5°C. Young virions (YOUNG VIR) were isolated from Ad2*ts*1-infected cells at 39.5°C. All virion structures were purified through three rounds of CsCl density centrifugation. Particles were disrupted by boiling in buffer containing SDS and reducing agent, and then their constituent polypeptides were separated by electrophoresis on a 10% polyacrylamide gel. The gel was blotted to nitrocellulose and probed, as described in the legend to Fig. 1, with a pool of monoclonal antibodies specific for the 52/55-kDa proteins (A) or with a polyclonal antibody prepared against Ad2 virions (B). The polyclonal antibody recognizes most Ad5 structural proteins, with the obvious exception of the hexon protein (II). An extract from Ad5*pm*382-infected cells (382 EXTR) is included in panel A as a positive control. This mutant overexpresses the 52/55-kDa proteins. Arrows in panel A designate the major 52/55-kDa protein species. Arrows in panel B designate major virion proteins. Molecular mass markers, shown as dashes between panels A and B, are, from the top, 92.5, 69, 46, and 30 kDa.

apparent molecular mass of 48 kDa. This makes sense since the L1 52/55-kDa open reading frame is predicted to encode a single 415-amino-acid product with a molecular mass of 47 kDa.

52/55-kDa proteins are components of assembling particles but not mature virions. With antibodies available, it was possible to determine whether the L1 52/55-kDa proteins were present within assembling adenovirus particles. Particles representing the various assembly intermediates—empty capsids, intermediate particles, and young virions—can be isolated by using wild-type and mutant viruses. Mature virions and empty capsids were purified from wild-type virus-infected (Ad5*wt*300 and Ad2) cells. Intermediate particles were isolated from cells infected with Ad5*ts*369 (mutation in the L1 52/55-kDa protein coding region [12]) and maintained at the nonpermissive temperature. Young virions were isolated from cells infected with Ad2*ts*1 (mutation in the L3 23-kDa protease coding region [32, 33]) and maintained at the nonpermissive temperature. The particles were purified and subjected to protein blot analysis. As shown in Fig. 2A, when particles (8.3×10^{10} per lane) were probed with a mixture of antibodies that recognize the L1 52/55-kDa proteins, the proteins were detected within Ad5*wt*300 and Ad2 empty capsids, Ad5*ts*369 intermediate particles, and Ad2*ts*1 young virions. The proteins were not detected within mature Ad5 or Ad2 virions, even when 10 times (8.3×10^{11} particles, Fig. 2A, 10 × Ad2 VIR or 10 × Ad5 VIR) or 60 times (5×10^{12} particles, data not shown) more virions were loaded per lane.

Several apparent breakdown products are detected by the antibodies to the 52/55-kDa proteins in empty capsids, intermediate particles, and virions but not within whole cell extracts or young virions (Fig. 2A). They may result from cleavage of the 52/55-kDa proteins at a late stage during encapsidation by the viral endoproteinase (2, 6, 9, 18). This proteinase cleaves a variety of virion proteins during encapsidation, and the 52/55-kDa proteins contain a consensus cleavage site for this proteinase between amino acids 351 and 352. It is possible that the major 40-kDa immunoreactive doublet present in empty capsids and intermediate particles is the 351-amino-acid product resulting from cleavage of the 52/55-kDa proteins. Since the young virions are produced from Ad2*ts*1-infected cells, which do not contain functional proteinase at the nonpermissive temperature, they would not be expected to contain putative cleavage products, and these products were not observed. The two smallest species detected during analysis of large quantities of virions result from cross-reaction of the antibodies with the pVII and VII core proteins, which are not present in empty capsids or intermediate particles that lack cores.

As a control, the same samples were probed with a polyclonal antiserum which recognizes Ad2 virion proteins (Fig. 2B). This serum does not recognize Ad5 hexon but does effectively recognize all other Ad5 virion components. This protein blot indicated that equal numbers of particles were loaded in all lanes and displayed the lack of protein V within intermediate particles and empty capsids, confirming that they do not contain cores. Young virions, however, do contain protein V and are enriched in precursor proteins such as preVI and preVII.

The analysis presented in Fig. 2 demonstrated that the 52/55-kDa proteins can be constituents of viral capsids but

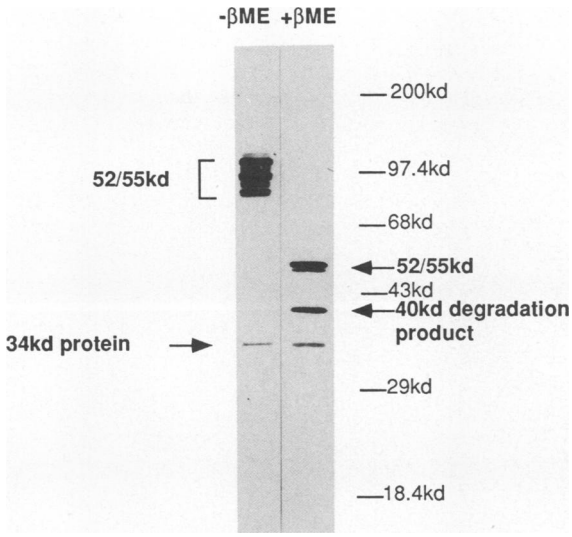

FIG. 3. Reducing agents affect the mobilities of 52/55-kDa proteins in polyacrylamide gels. Ad5*ts*369 intermediate particles, purified through three rounds of CsCl equilibrium density centrifugation, were mixed with buffer in the presence (+βME) or absence (−βME) of 700 mM β-mercaptoethanol. The samples were boiled for 5 min and separated on a 10% polyacrylamide gel. Western blot analysis was performed, as described in the legend to Fig. 1, by using a mixture of monoclonal antibodies to the 52/55-kDa proteins, whose positions are indicated by a bracket on the left and an arrow on the right. The 40-kDa breakdown product is indicated by an arrow. A presumptive cross-reacting protein of approximately 34 kDa, unaffected by the reducing agent treatment, is indicated on the left. Molecular masses of marker proteins (indicated by dashes) are shown.

did not directly address the possibility that the proteins might also associate with DNA-containing core structures. To test this possibility, cores were isolated from young virions by sedimentation of pyridine-treated particles in 10 to 25% sucrose gradients. The fractions from these gradients were subjected to protein blot analysis, and no 52/55-kDa proteins were detected in fractions containing cores. Rather, the 52/55-kDa proteins fractionated at the top of the gradient with the capsid proteins that were released by pyridine treatment (data not shown). This experiment, of course, does not rule out a weak, pyridine-sensitive interaction of the 52/55-kDa proteins with the core.

We conclude that the 52/55-kDa proteins are transient constituents of the capsid during the assembly process. It seems likely that they are cleaved, with the cleavage process beginning fairly early during the encapsidation process.

52/55-kDa proteins appear to exist as disulfide-linked homodimers. The mobilities of 52/55-kDa proteins during polyacrylamide gel electrophoresis are different in the presence and absence of β-mercaptoethanol (Fig. 3). 52/55-kDa protein samples lacking a reducing agent migrate at a molecular mass approximating that of a homodimer. 52/55-kDa proteins from all virion assembly intermediates as well as from cell extracts behave in this fashion (data not shown). The change in mobility in response to reducing agent is unique to the 52/55-kDa proteins; no other virion component had an altered mobility in the absence of reducing agent (II, IIIa, IV, V, VI, VII, and VIII were monitored; data not shown).

The 40-kDa product, present in intermediate particles and empty capsids (see Fig. 2A), is also affected by the reducing agent. In fact, the sizes of the three main bands observed in the absence of reducing agent suggest that they are full-length protein:full-length protein, full-length protein:40-kDa protein, and 40-kDa protein:40-kDa protein dimers, with the less prominent intermediate bands likely representing different phosphoforms. The anti-52/55-kDa monoclonal antibodies also react at low levels with a 34-kDa species. This protein does not alter its mobility in response to reducing agent. Possibly, this protein species is a cleavage product that lacks the dimerization domain; alternatively, the 34-kDa polypeptide may not be derived from the L1 52/55-kDa proteins.

TABLE 2. L1 52-55-kDa-protein copy number in virion assembly structures

Expt no.	Protein copy no. in:		
	Young virions[a]	Intermediates[b]	Empty capsids[c]
1	1.7, 2.2	4.9, 3.6, 3.5	56
2	1.0, 1.0	Not done	Not done
3	1.8, 2.2	Not done	Not done

[a] The number of 52/55-kDa-protein molecules per young virion was determined in three independent experiments. HeLa cells were infected with Ad2*ts*1 and labeled with a mixture of [^{35}S]Met and [^{35}S]Cys, and young virions were purified. Portions of the purified particles were disrupted, and then either total virion proteins or quantitatively immunoprecipitated 52/55-kDa proteins were separated by electrophoresis. Bands corresponding to hexon, protein V, and 52/55-kDa proteins were excised, radioactivity was quantified and normalized for the Met plus Cys content of each protein, and the normalized number was used to calculate copy number relative to the hexon protein (first number tabulated for each experiment) and protein V (second number). There are 720 and 180 molecules of hexon and protein V, respectively, per particle.

[b] The 52/55-kDa-protein copy number per intermediate particle was determined from three separate immunoprecipitations relative to hexon protein only, since these particles do not contain protein V. The estimate includes only intact 52/55-kDa proteins.

[c] The 52/55-kDa protein copy number per empty capsid was determined relative to hexon protein. The estimate includes only intact 52/55-kDa proteins.

Copy number of 52/55-kDa proteins in assembling particles. Quantitative immunoprecipitation experiments were undertaken to determine the number of 52/55-kDa protein molecules found within young virions, intermediate particles, and empty capsids (Table 2). Particles labeled with [^{35}S]methionine plus [^{35}S]cysteine were isolated and disrupted with guanidinium hydrochloride (disruption of particles was required to give antibodies access to the 52/55-kDa proteins); multiple, sequential cycles of immunoprecipitation were carried out to ensure that all 52/55-kDa protein was captured. To determine the relative number of 52/55-kDa protein copies, the radioactivity within the precipitated 52/55-kDa protein band was compared with that within both protein V and hexon, whose copy numbers are known. Taking into account the number of cysteines and methionines within each protein, it was determined that, on the average, one to two molecules of 52/55-kDa protein are found within a young virion.

Similar quantitation experiments were attempted with intermediate particles and empty capsids, but since there is not a full complement of hexon within these particles and breakdown products of the 52/55-kDa proteins are present (see Fig. 2A), there is a greater margin of error. Intermediate particles appeared to have more full-length 52/55-kDa-protein molecules than young virions, but the number was much higher for empty capsids, about 56 copies per particle (Table 2). The calculation for the 52/55-kDa-protein copy number in

empty capsids and intermediate particles did not include immunologically related but less-than-full-length molecules. If these putative cleavage products (i.e., the 40-kDa doublet discussed above) are included, then the estimate for empty capsids exceeds 100 copies per particle.

Immunofluorescent localization of the 52/55-kDa proteins within infected cells. One model of virus assembly couples encapsidation of the viral DNA with its replication (34). This model predicts that assembly sites and replication sites coincide since DNA is inserted into a capsid as it is replicated. The 52/55-kDa proteins should be a specific marker for assembling virions as judged by the fact that they were found within all virion assembly structures. To determine whether sites of virus assembly coincided with sites of DNA replication, the location of the L1 52/55-kDa proteins was compared with that of the E2 72-kDa protein, a major component of viral replication centers (21, 23, 31), in a double antibody labeling experiment.

HeLa cells were infected with Ad5*wt*300, Ad5*pm*382 (overexpresses wild-type 52/55-kDa protein), Ad5*ts*369 (expresses mutant 52/55-kDa protein), wild-type Ad2, or Ad2*ts*1 (expresses mutant L3 23-kDa proteinase). Permeabilized, infected HeLa cells were probed at 24 h postinfection with both anti-52/55-kDa-protein monoclonal antibody 16 (recognized by a fluorescein-coupled second antibody; Fig. 4A) and an anti-72-kDa DNA-binding protein antibody (recognized by a rhodamine-coupled second antibody; Fig. 4B). Identical results were observed with the other 52/55-kDa-protein antibodies (data not shown). In addition, the cellular DNA was stained with the fluorescent dye DAPI (Fig. 4C). The stage of infection can be monitored by assessing the integrity of nucleoli by phase-contrast illumination (Fig. 4D). At early stages, nucleoli exhibit their characteristic morphology (Fig. 4D, Nu), but later in infection these structures are no longer apparent (21). Also, at later stages the nuclei are distended, with an irregular border.

Replication centers are detected as brightly staining bodies recognized by the anti-72-kDa DNA-binding protein antibody (Fig. 4B; arrows). The replication centers appear as round bodies early in infection which grow and then fuse into large replicating regions later in infection (Fig. 4A; cell marked with asterisk). As has been previously reported (21), the round replication centers colocalize with dense structures (compare arrows in Fig. 4B and D).

There are two types of 52/55-kDa protein staining (Fig. 4A). Earlier in infection, as shown by the distinct nucleoli (Nu) in the corresponding phase-contrast micrograph in Fig. 4D, brightly staining spots are evident (arrowheads). These spots appear to be tiny rim-stained spheres, as they exhibit a circular but hollow fluorescence image. This spherical fluorescence can be visualized more readily by focusing through the depth of the cell. In addition to the bright spots, there is a diffuse nuclear stain. This stain is excluded from nucleoli and is not homogeneously dispersed throughout the nucleus. Upon close examination, the diffuse stain appears finely punctate. The spots of 52/55-kDa protein do not colocalize with replication centers (compare arrowheads in Fig. 4A with arrows in B). Rather, they seem to colocalize with extremely dense bodies visible in Fig. 4D (arrowheads). At later stages in infection, the brightly staining regions of 52/55-kDa protein appear to be at the periphery of the nucleus (Fig. 4A; cell marked with asterisk). By 36 h postinfection, most infected cells exhibit bright staining at the nuclear periphery (data not shown). Interestingly, at late times, the 52/55-kDa protein staining seems to be found in regions which exclude both the 72-kDa DNA-binding protein and DAPI DNA staining (compare cells marked with asterisks in Fig. 4A to C). This location is consistent with electron microscopic studies which have shown that at later stages of infection, the assembled virions are found at the periphery of the nucleus (12, 23, 31). It is expected that such a region

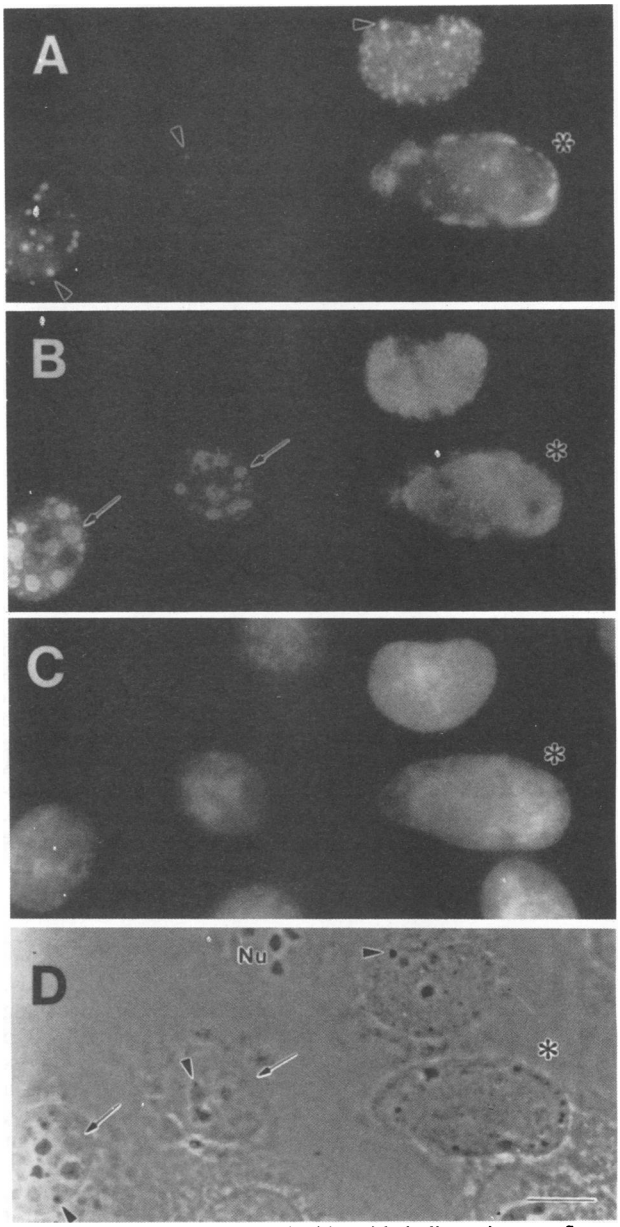

FIG. 4. Dark structures coincide with indirect immunofluorescent staining for the 52/55-kDa proteins in Ad5*pm*382-infected cells. HeLa cells were infected at a multiplicity of 25 PFU per cell and processed 24 h later for indirect immunofluorescence with anti-52/55-kDa-protein monoclonal antibody 16 (A) or antibody specific for the E2 72-kDa DNA-binding protein (B). The corresponding cells stained with DAPI are shown in panel C, and their phase-contrast image is shown in panel D. Nu indicates nucleoli; arrowheads indicate dark structures recognized by the antibody to 52/55-kDa protein; arrows indicate dense vesicular structures recognized by the 72-kDa protein antibody; and asterisks indicate cells at late stages of infection. Bar, 10 μm.

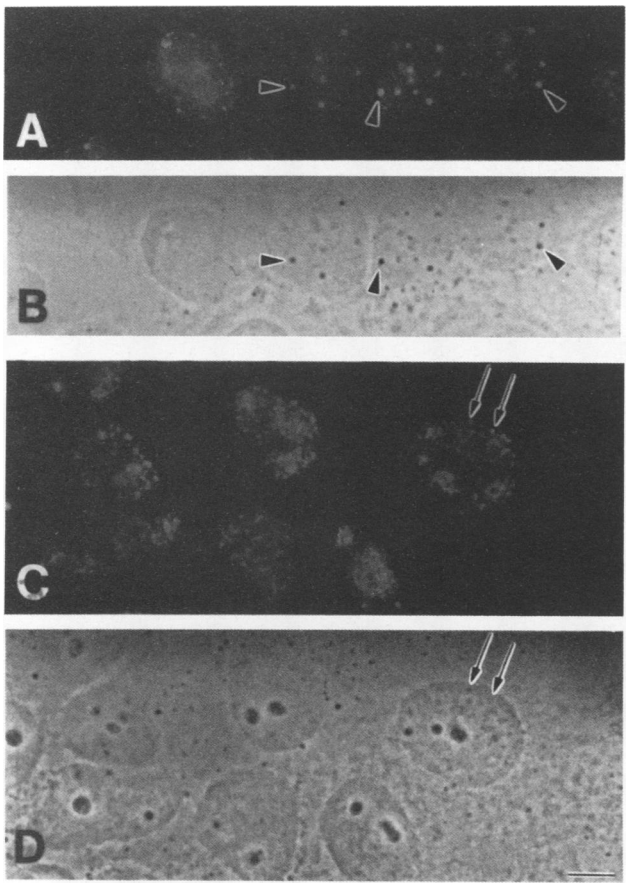

FIG. 5. L1 52/55-kDa-protein and E2 72-kDa-protein localization within Ad5*wt*300-infected cells is not altered by extraction. A monolayer HeLa cell culture was infected at 39.5°C with Ad5*wt*300 at a multiplicity of 25 PFU per cell. Twenty-four hours later, the cells were extracted, fixed, and stained with anti-52/55-kDa-protein monoclonal antibody 16 (A) or the anti-72-kDa-protein antibody (C) and prepared for indirect immunofluorescence analysis. Phase-contrast micrographs corresponding to the fluorescent images are shown in panels B and D, respectively. Panels A to D are at the same magnification. Bar, 10 μm.

excludes both the 72-kDa DNA-binding protein and cellular chromatin.

Given the assumption that 52/55-kDa proteins colocalize with assembling virions within the nucleus, we can conclude that virus assembly begins at multiple sites which expand or coalesce as the infection proceeds, eventually filling much of the nuclear periphery. The majority of presumptive packaging centers marked by the presence of L1 52/55-kDa proteins clearly do not coincide with replication centers.

Immunoelectron microscopic localization of 52/55-kDa proteins. To better elucidate the relationship between the brightly staining bodies observed by immunofluorescence and the sites of virus assembly, immunoelectron microscopic studies were undertaken. Both anti-52/55-kDa-protein antibody 16 and the anti-72-kDa DNA-binding protein antibody were utilized in these experiments. For this analysis, Ad5*wt*300-infected HeLa cells were extracted and infiltrated with antibodies before they were embedded in resin and sectioned. As judged by immunofluorescence, the extraction procedure did not affect either 72-kDa-protein or 52/55-kDa-protein localization within infected cells (compare Fig. 5A to D with Fig. 4). Mock-infected cells, included as a negative control, did not label with the antibodies (data not shown).

The portion of a whole cell shown at lower magnification in the electron micrograph displayed in Fig. 6A exemplifies the complexity of the infected nucleus; numerous bodies of various staining densities are found. Unlike whole cells, the extracted cells embedded in resin lack a visible cytoplasm, nucleoli, and crystals of hexon. Chromatin makes up the darkly staining fibrils found near the periphery of the nucleus, as determined by differential staining experiments (19). The multiple 70-nm hexagonal structures are virions. Virions appear as both very dark or intermediately staining structures. Dark particles are complete, DNA-containing virions. Light particles could be assembly intermediates with no DNA or complete virions sectioned so that DNA was not accessible to the stain. The very lightly staining fibrous inclusions are the sites of viral DNA replication (determined by electron microscopic autoradiograms of [^{3}H]thymidine-labeled infected cells [17, 26, 27]). As expected, the lightly staining fibrous inclusions contain 72-kDa DNA-binding protein, as they are well decorated by gold beads when incubated with the anti-72-kDa DNA-binding protein antibody (arrowheads in Fig. 6E).

The anti-52/55-kDa-protein antibody recognizes a series of small structures within the infected nucleus, giving three main patterns of labeling with gold beads (Fig. 6B to D). The arrowhead in Fig. 6B marks the gold-labeled structure which we have termed a loading center. Found in well-extracted nuclear regions, these structures are always surrounded by numerous virions. Such a structure has not been described previously, and it is distinct from chromatin (darkly staining masses) and replication centers (lightly staining fibrils; see gold-labeled structures in Fig. 6E). It is possible that the presence of the 52/55-kDa-protein-specific antibodies stabilizes or compacts the loading center such that it becomes a better-defined structure. This change in structure upon antibody binding is evident for the replication centers bound by the anti-72-kDa DNA-binding protein antibody (compare gold-labeled structures in Fig. 6E with similar structures in Fig. 6A to C). Antibody-bound replication centers always appear less fibrous and more compact. The antibody to 52/55-kDa protein was not observed to label replication centers (lightly staining fibrillar structures in Fig. 6A to C; see gold-labeled structures in Fig. 6E), which is in agreement with the fluorescence data.

The anti-52/55-kDa-protein antibodies also recognize circular, punctate, densely staining bodies (Fig. 6C). These bodies most likely make up the brightly staining structures observed by immunofluorescence (Fig. 4A and 5A). The peripheral gold labeling observed here is in agreement with the rimlike fluorescent staining of these bodies (Fig. 4A). The peripheral staining of the antibody is most likely due to the impenetrable nature of this structure. In some cases, these bodies are associated with virions. The punctate appearance of these bodies is curious but may in fact be an artifact of antibody binding. 52/55-kDa protein bodies which are not antibody labeled display a homogeneous staining pattern (compare undecorated dense body in Fig. 6E with gold-bead-labeled structure in Fig. 6C).

The final anti-52/55-kDa-protein labeling pattern, shown in Fig. 6D, most likely reflects the diffuse staining component observed by immunofluorescence. Throughout the infected nucleus, the 52/55-kDa proteins appear to be present in close association with lightly staining 70-nm particles. Visualized as small clusters of gold beads, the labeling pattern often

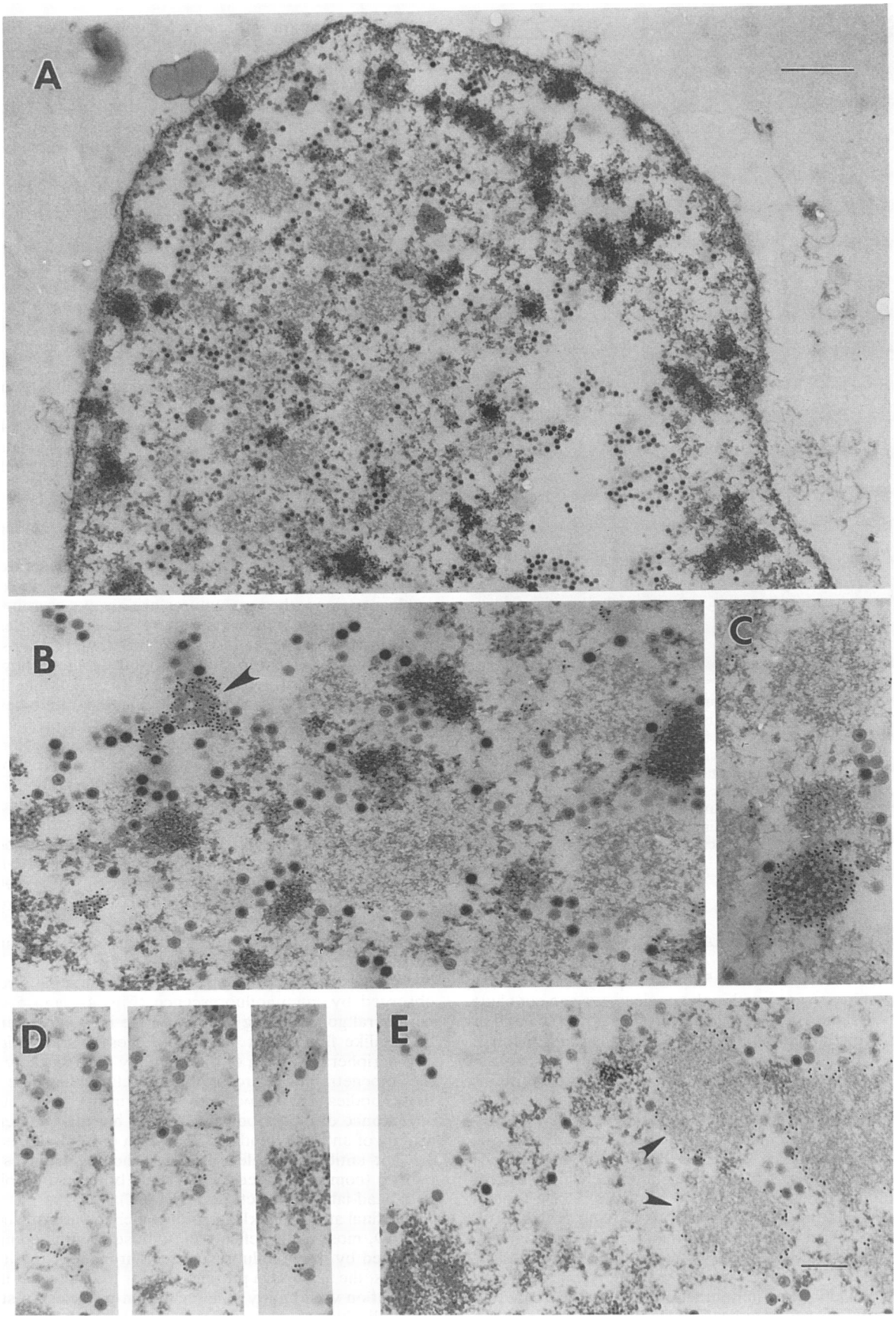
A
B
C
D
E

FIG. 6. Immunogold labeling of extracted Ad5*wt*300-infected cells shows a close association of the L1 52/55-kDa proteins with incomplete viral particles. Infected cells prepared as described in the legend to Fig. 5 were incubated with 52/55-kDa-protein-specific monoclonal antibody 16 (A to D) or E2 72-kDa-protein-specific monoclonal antibody B6-8 (E); incubation was followed by reaction with immunogold-conjugated (10-nm beads) goat anti-mouse IgG (fc), prior to embedment in Epon. (A) Low-magnification survey. Bar, 1 μm. (B) Loading center recognized by the 52/55-kDa-protein-specific antibody (arrowhead). (E) DNA replication centers recognized by the 72-kDa-protein-specific antibody (arrowheads). Panels B to E are at the same magnification. Bar, 0.25 μm.

appears to be associated with a single corner of the virion particle. This close association with lightly stained, possibly incomplete virus particles is consistent with a role for the 52/55-kDa proteins in virus assembly.

DISCUSSION

The production of a series of monoclonal antibodies (Table 1) has enabled us to gain several new insights into the L1 52/55-kDa proteins and their function in virion assembly. First, the two proteins are differentially phosphorylated versions of a single polypeptide with an apparent molecular mass of 48 kDa (Fig. 1). The apparent molecular mass of the dephosphorylated protein fits well with the size of the 52/55-kDa-protein open reading frame, and this observation lays to rest earlier speculation that the 52/55-kDa proteins might arise from posttranslational cleavage or result from an undiscovered alternative splicing event. The phosphorylation state of the 52/55-kDa proteins does not appear to change during infection (data not shown), and the function of the differential phosphorylation, if any, remains unclear. Second, the 52/55-kDa proteins appear to exist as disulfide-linked homodimers (Fig. 3). These proteins are unique among adenovirus structural proteins in their ability to form a protein complex that is sensitive to reducing agents (data not shown). The linkage presumably involves the single cysteine residue present in each copy of the 52/55-kDa protein. Third, the 52/55-kDa proteins were not detected in mature virions, but they are present in all putative virion assembly structures: empty capsids, intermediate particles, and young virions (Fig. 2). The 52/55-kDa-protein copy number is 1 to 2 in young virions and 50 to 100 in empty capsids (Table 2). Fourth, the 52/55-kDa proteins are localized within nuclear structures that are morphologically distinct from viral replication centers (Fig. 4 to 6). If one assumes that virus assembly occurs in the vicinity of the bulk of the 52/55-kDa proteins, then assembly occurs at a location distinct from that of replication within the infected nucleus. This result argues against assembly models in which viral DNA is presumed to be replicated into empty capsids (34), since such models require that replication and assembly occur in close proximity. Fifth, immunogold electron microscopy visualized discrete structures containing the 52/55-kDa protein that may be assembly or loading centers within the nucleus (Fig. 6).

We were unable to detect a pyridine-resistant association of the proteins with the viral chromosome (data not shown), as is the case for the core proteins (proteins V, VII, and μ). The 52/55-kDa proteins are clearly present in empty capsids, but none of the monoclonal antibodies could precipitate the 52/55-kDa proteins without prior dissociation of the structure (data not shown). Since all of the antibodies recognize the N-terminal domain of the proteins, it seems likely that this portion of the proteins is located internally and not exposed on the particle surface. Perhaps the 52/55-kDa proteins are located entirely on the inside surface of the capsid.

What is the function of the L1 52/55-kDa proteins during virion assembly? By comparison with other virus systems, one can propose four possible roles. The 52/55-kDa proteins could play a structural role as scaffolding proteins; they might function to mediate recognition between capsid and chromosome at the initiation of encapsidation; they could play a catalytic role in the compaction of the viral chromosome; or they could form a portal structure through which the viral DNA must enter the virion capsid. Loss of any one of these four functions could disrupt virion assembly and generate assembly intermediates such as those which accumulate in the absence of 52/55-kDa protein function (12).

At present, we favor the hypothesis that the proteins perform a scaffolding function during virion assembly. Scaffolding proteins have been described for a variety of bacteriophage systems (for a review, see reference 4). The study of mutant bacterial viruses by using in vitro assembly systems has revealed that scaffolding proteins play key roles in the formation of functional capsids, and mutant scaffolding proteins can cause accumulation of nonfunctional empty capsid intermediates. On the order of several hundred molecules of scaffold protein are present in each newly assembled capsid, and they remain associated with the particles until they are released or cleaved during maturation of the viral particle. These characteristics fit well with what we know of the L1 52/55-kDa proteins. The adenovirus proteins are present in relatively high copy number in empty capsids, but they are not in virions (Table 2), and it is likely that the proteins are cleaved during virion assembly (Fig. 2 and 3). Further, the mutant 52/55-kDa proteins produced in Ad5*ts*369-infected cells at the nonpermissive temperature generate nonfunctional intermediate particles (12). The high copy number of 52/55-kDa proteins in empty capsids is consistent with a scaffolding role for the proteins during assembly of the capsid.

Even though DNA replication and virus assembly appear to occur in separate nuclear compartments (Fig. 4 and 6), we suspect that the capsid does not assemble as an independent structure, separate from the viral chromosome. If empty capsids were able to assemble in the absence of viral DNA, then substantial quantities of the structure would accumulate in cells infected with Ad5*dl*309-A5, a deletion mutant with a crippled *cis*-acting packaging element whose DNA is assembled into virions very inefficiently (13). However, essentially no empty capsids accumulate in Ad5*dl*309-A5-infected cells even though normal quantities of viral polypeptides are present (11a), suggesting that capsid formation occurs in association with a DNA molecule carrying a functional *cis*-acting packaging element. A rate-limiting step appears to occur after assembly of capsids, which results in accumulation of intermediate particles, especially in group B adenoviruses (9, 30). Nonfunctional intermediate particles also accumulate in the presence of mutant 52/55-kDa proteins (12). Perhaps capsids are incorrectly assembled under these conditions, and this leads to a failure in the assembly process, with accumulation of nonfunctional intermediate particles.

Although it seems likely that the 52/55-kDa proteins perform a scaffolding function during capsid formation, we cannot definitively rule out other possible functions during virion assembly. In fact, it is possible that the proteins are multifunctional and perform several functions during assembly. For example, a few copies of the protein could presumably serve to mediate a recognition event between proteins assembled at the *cis*-acting packaging site on the viral chromosome (for examples, see references 11, 13, and 30) and the empty capsid.

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