Nuclear Assembly of Polyomavirus Capsids in Insect Cells Expressing the Major Capsid Protein VP1

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Polyomavirus normally assembles in the nucleus of infected mouse cells. Sf9 insect cells expressing the polyomavirus major capsid protein VP1 were examined by electron microscopy. Capsidlike particles of apparently uniform size were found in the nucleus. Immunogold electron microscopy demonstrated abundant VP1 in the cytoplasm which was not assembled into any recognizable higher-order structure. Cytoplasmic VP1 assembled after the cells were treated with the calcium ionophore ionomycin. Purified VP1 aggregates were shown by negative staining and cryoelectron microscopy to consist predominantly of particles similar to the empty T = 7 viral capsid. Thus, polyomavirus VP1 can assemble in vivo into capsids independent of other viral proteins or DNA. Nuclear assembly may result from increased available calcium in this subcellular compartment.

The capsid proteins of papovaviruses are synthesized in the host cytoplasm and then transported to the cell nucleus where they assemble on the viral minichromosome to produce the virion structure (45). The polyomavirus capsid is built from 72 pentamers of the VP1 protein, which form three different types of connections in the T = 7d icosahedral surface lattice (39), in contradiction to the theory that quasi-equivalent bonding should be conserved in regular icosahedral virus capsids (11). In vitro self-assembly of bacterially expressed recombinant VP1 into capsidlike aggregates (41, 42) has demonstrated that switching of the bonding specificity to form the nonequivalent connections is an inherent property of the VP1 pentamers. In vitro, addition of low concentrations of calcium at physiological ionic strength and pH leads to self-assembly of the recombinant protein (42), supporting the important structural role of calcium previously indicated by virion dissociation studies (8, 13, 19). The atomic structure of VP1 in the closely related simian virus 40 capsid which has been determined from 0.38-nm resolution virus crystal diffraction data (28b) is now revealing the bonding interactions between the subunits and the calcium-binding sites in the intact virus structure.

Characterization of VP1 assembly in vitro can elucidate only selected aspects of the in vivo control of virus assembly, and some control mechanisms may only be studied in vivo. For example, phenotypes of temperature-sensitive mutations in the simian virus 40 VP1 gene (6, 7) support the view, developed from studies isolating subvirion intermediates (4, 16, 20, 22), that capsid proteins are incrementally added to the viral minichromosome, rather than the viral genome being injected into a preformed capsid as occurs in bacteriophage assembly. The transforming genes of the polyomavirus also play a role in virus assembly as shown by the host-range nontransforming (*hr-t*) polyomavirus mutants (5), defective in middle and small T antigens, which are inefficient in packaging the viral minichromosome into complete virions in nonpermissive mouse 3T3 cells (21). Assembly in vivo is also affected with simian virus 40 agnogene

mutant viruses (2, 36, 43). These mutant viruses grow poorly on certain cell types and appear to be defective in the nuclear transport of VP1 (10, 40). Second-site revertants of the agnogene mutation map to VP1 (32), and the agnoprotein may directly interact with VP1 to prevent premature cytoplasmic aggregation of VP1 pentamers, thus facilitating their nuclear localization (3).

Recent studies using baculovirus expression of RNA viral coat proteins in Sf9 insect cells demonstrate that in vivo virus assembly can be assayed under conditions in which the entire viral genome is not present and only selected proteins are expressed. For example, assembly of the human immunodeficiency virus type 1 precursor Pr55^{gag} protein (25), the bovine immunodeficiency virus Pr53^{gag} protein (38), type 3 poliovirus (P3/Leon/37 strain) (47), and bluetongue virus (17, 18, 29) has been studied by baculovirus expression. Such systems now allow characterization of individual stages in RNA virus morphogenesis. As a test of the intrinsic in vivo assembly properties of a DNA virus coat protein, we expressed the polyomavirus capsid protein VP1 in insect cells using a baculovirus vector (9, 30) and examined the self-assembly of this protein by electron microscopy of sectioned cells and purified preparations. Using this technique, we investigated the intracellular localization of VP1 in infected cells and the state of its association.

MATERIALS AND METHODS

Expression of VP1 in Sf9 cells. A 2-kb *EcoRV*-to-*PvuII* fragment was isolated from polyomavirus DNA. *Bam*HI linkers were ligated, and the resulting fragment was cloned into the *Bam*HI site of pVL941 derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV) (30). The *EcoRV* site is 34 bp 5' to the VP1 ATG, and proper orientation with respect to the polyhedrin gene promoter in pVL941 was determined by subsequent digestion with *Hind*III. Recombinant baculoviruses were generated by cotransfecting *Spodoptera frugiperda* (Sf9) cells with AcNPV viral DNA and pVL941-VP1. Plaque transfer was performed as described previously (9, 44), and the nitrocellulose filters were probed with a nick-translated ³²P-labeled

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HindIII-to-*PvuII* fragment of the polyomavirus genome (31). Hybridizing plaques were purified to yield AcNPV-VP1.

Immunoblot analysis. Cells (3×10^6 to 4×10^6) from a 60-mm plate were removed with the medium by pipetting and washed twice with phosphate-buffered saline (PBS) by centrifugation at $2,000 \times g$, and the final cell pellet was resuspended in 1 ml of PBS. A 200- μ l sample of this resuspension was centrifuged at $10,000 \times g$ for 5 min, solubilized in 100 μ l of sodium dodecyl sulfate (SDS) sample buffer, and used for each lane of electrophoresis on an SDS-10% polyacrylamide gel (27) (i.e., each lane represents the total protein from approximately 6×10^5 to 8×10^5 cells). Western transfer (immunoblotting) (46) was performed with a dry blot apparatus (Hoeffer) and developed with a polyclonal rabbit anti-VP1 antiserum (20a) made against recombinant VP1 protein (28), using a goat antirabbit alkaline phosphatase-conjugated second antibody (Promega).

Purification of VP1 protein from AcNPV-VP1-infected Sf9 cells. The cell pellet from 1 liter of AcNPV-VP1-infected Sf9 cells was frozen, thawed, and lysed by douncing 10 times with a B pestle in 50 ml of 250 mM NaCl-1 mM EDTA-15 mM 2-mercaptoethanol-5% glycerol-1 mM phenylmethylsulfonyl fluoride-0.05% sodium deoxycholate-20 mM Tris (pH 7.9). The homogenate was centrifuged at $10,000 \times g$ for 20 min, and Polymin P was added to the supernatant to a final concentration of 3.5%. The resulting precipitate was extracted twice with buffer B plus 1 M NaCl (1 M NaCl, 15 mM 2-mercaptoethanol, 5% glycerol, 0.1 mM EDTA, 20 mM Tris [pH 7.2]), and the combined supernatants were precipitated by adding solid ammonium sulfate to 35% saturation. The ammonium sulfate precipitate was dialyzed against buffer B plus 100 mM NaCl, and the soluble fraction after dialysis was chromatographed on a DE-52 cellulose column. The flowthrough plus one column volume were collected and precipitated with solid ammonium sulfate to 35% saturation. The precipitate was dialyzed against buffer B plus 100 mM NaCl and then applied to a P-11 phosphocellulose column that was washed with buffer B plus 250 mM NaCl and eluted with buffer B plus 1 M NaCl.

Two-dimensional gel electrophoresis and phosphoamino acid analysis. Samples for two-dimensional gels (final volume, 50 μ l) were prepared by the addition of 10% SDS to 0.5%, EDTA to 1 mM, and 2-mercaptoethanol to 150 mM. The samples were then heated to 100°C for 5 min and cooled to room temperature, and solid urea was added to saturation. Nonidet P-40 was then added to 2%. Electrophoresis was performed as previously described (37). Phosphoamino acid analysis was performed after $^{32}\text{P}_i$ labeling of AcNPV-VP1-infected Sf9 cell monolayers in phosphate-free Grace's medium for 4 h at 2 mCi/ml or of polyomavirus (wild-type strain RA)-infected A31 mouse fibroblasts as previously described (23). Cells were lysed in radioimmunoprecipitation assay buffer, and VP1 was immunoprecipitated (23). After 10% polyacrylamide gel electrophoresis (PAGE) and electrophoretic transfer to Immobilon paper, the VP1 band was excised, hydrolyzed, and subjected to thin-layer chromatography (33).

Electron microscopy of thin sections. Monolayers were prepared for thin sectioning by first being fixed in 2.5% glutaraldehyde in PBS for 1 h at 4°C. Further preparation for sectioning was done as previously described (14), using 1% osmium tetroxide as a postfixative. Serial ultrathin (60-nm) sections were cut true to the plane of the dish and mounted on 300-mesh grids. Monolayers for immunolabeling were first fixed in 0.001% glutaraldehyde-2% paraformaldehyde. Dishes were then scraped, and the cells were pelleted,

resuspended in 3% gelatin in PBS, solidified on ice, infused with 2.3 M sucrose, and subsequently prepared and sectioned (48). Sections for immunolabeling were blocked for 30 min in normal goat serum (5% in PBS-bovine serum albumin [BSA]) and then washed three times in PBS-BSA. Sections were then incubated with rabbit anti-recombinant VP1 antiserum for 30 min at 22°C, washed three times as before, incubated with a 5-nm gold-conjugated goat antirabbit second antibody (Janssen), and then washed again three times. Sections were examined with a Philips EM 300 electron microscope.

Ionomycin treatment. Ionomycin at a stock concentration of 10 mg/ml (13.4 mM) in ethanol was assayed for cell toxicity by trypan blue exclusion. A 1-h exposure of cells at 72 h postinfection (p.i.) to 5 μ M drug gave 33% survival compared with 82% in untreated controls. For electron microscopy, after the 1-h exposure, the cells were gently washed twice with PBS and then fixed directly on the plate with 2.5% glutaraldehyde in PBS.

Purification of particles. At 72 h p.i., cells and media from five 100-mm plates were removed by pipetting, and the cells were washed twice with PBS plus 1 mM calcium and once with buffer A (1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM KCl, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] (pH 7.9) by centrifugation at $2,000 \times g$. The cell pellet was resuspended in 1 ml of buffer A and processed as described previously (22). The neuraminidase-treated supernatant was saved, and the pellet was extracted further with 1 ml of buffer A containing 1% Nonidet P-40. After Dounce homogenization and centrifugation at $3,000 \times g$, this second supernatant was also saved. A 400- μ l sample of each supernatant was loaded on separate 5 to 30% sucrose gradients in buffer A with 1 M NaCl. The gradients were centrifuged for 70 min at 39,000 rpm in an SW40 rotor (Beckman) at 4°C. Twenty-four fractions from each gradient were collected from below (0.3 to 0.5 ml). Fractions were assayed for VP1 by both immunoblotting and hemagglutination of sheep erythrocytes (22). The VP1 protein and hemagglutination activity peaked broadly around 140S in both preparations. These fractions were pooled from both gradients, dialyzed against 250 mM NaCl-20 mM HEPES [pH 7.2]-1 mM CaCl_2 -1 mM MgCl_2 , and concentrated by Centricon 100 (Amicon) filtration.

Electron microscopy of purified particles. Electron micrographs were recorded with Philips EM 301 and EM 420 electron microscopes operating at 80 and 100 kV, respectively. Preparation of grids of negatively stained specimens was performed as previously described (41). The sample for cryoelectron microscopy was embedded in a thin layer of vitreous ice by a quick-freezing procedure (15). The frozen specimens were transferred under liquid nitrogen to a cryo-specimen holder (Gatan) and examined in a Philips EM 420 electron microscope at a temperature of -170°C . The images were recorded with low irradiation (≈ 300 electrons per nm) and 1.0- μ m underfocus.

RESULTS

Expression of polyomavirus VP1 gene in insect cells. The VP1-coding sequence was cloned into the *Bam*HI site of the transfer vector pVL941 derived from AcNPV, and the pVL941-VP1 plasmid DNA was cotransfected into *S. frugiperda* (Sf9) insect cells with purified AcNPV DNA (44). Recombinant AcNPVs containing VP1 sequences were screened by plaque hybridization with a VP1-specific probe. Positive plaques were purified and tested for protein expres-

FIG. 1. SDS-PAGE analysis of VP1 protein expressed in Sf9 cells 72 h after infection with AcNPV-VP1. (A) Coomassie blue-stained gel of AcNPV-VP1-infected (lane 1), AcNPV-infected (lane 2), and mock-infected (lane 3) Sf9 cell extracts; (B) the same samples analyzed after immunoblotting with an anti-VP1 antibody. (C) Coomassie blue-stained gel of VP1 fractions purified from the AcNPV-VP1-infected Sf9 cells: lane 1, insoluble precipitate before DEAE chromatography; lane 2, DEAE column flowthrough; lane 3, phosphocellulose-purified VP1. Lane S, molecular weight standards (panels A and B with prestained markers, panel C with poststained markers).

sion by immunoblotting (46). Shown in Fig. 1 is the SDS-PAGE analysis of the VP1 protein produced after infection of Sf9 cells with the recombinant AcNPV-VP1. A prominent Coomassie blue-stained band (Fig. 1A; approximately 5 to 8% of total protein) is seen in the AcNPV-VP1-infected whole-cell extracts at a position corresponding to that of immunoreactive VP1 (Fig. 1B). Maximal VP1 accumulation occurred at 72 h p.i.

The VP1 protein was purified from the AcNPV-VP1-infected Sf9 cells (Fig. 1C) by a protocol modified from that used to purify VP1 after expression in *Escherichia coli* (28). Approximately 3 mg of near-homogeneous VP1 protein was obtained from 1 liter of AcNPV-VP1-infected Sf9 cells. Two-dimensional gel electrophoresis of the purified VP1 demonstrated one major and two minor VP1 isoelectric-focusing subspecies (data not shown) and was similar to the two-dimensional gel appearance of VP1 from virions (21, 23). In contrast, VP1 purified after expression in *E. coli* is essentially unmodified (28). Several of the modified subspecies of VP1 in polyomavirus-infected mouse cells result from phosphorylation (23). In vivo $^{32}\text{P}_i$ labeling of AcNPV-VP1-infected Sf9 cells demonstrated that VP1 was phosphorylated to a similar extent as VP1 from polyomavirus-infected mouse cells, and phosphoamino acid analysis demonstrated modification of both serine and threonine residues (Fig. 2). However, the stoichiometry of threonine-to-serine phos-

phorylation in the baculovirus-expressed VP1 (Fig. 2B; $\approx 80\%$ serine:20% threonine) differed from the ratio seen in VP1 from polyomavirus-infected cells (Fig. 2A; $\approx 70\%$ threonine:30% serine [23]). The stoichiometry of threonine-to-serine VP1 phosphorylation seen in AcNPV-VP1-infected Sf9 cells resembles that seen in *hr-t* mutant polyomavirus infection of nonpermissive mouse cells (23).

VP1 expressed in Sf9 cells assembles into viral capsidlike particles. Electron micrographs of thin sections of AcNPV-VP1-infected Sf9 cell monolayers fixed at 72 h p.i. (Fig. 3) show that distinct capsidlike particles accumulated in the nucleus near the inner nuclear membrane. No such structures were seen in mock- or AcNPV-infected Sf9 cells. Particle dimensions were determined by measuring the diameters of 400 individual particles, scaled by magnification standards. The particles appeared nearly homogeneous, with a diameter of 35 ± 5 nm (standard deviation) uncorrected and 40.5 ± 6 nm (standard deviation) corrected for dehydration shrinkage of the specimen by using the 24-nm cross-sectional diameter of cytoplasmic microtubules as an internal control. The 35-nm dimension is consistent with that seen in thin sections of polyomavirus-infected cells which have been examined by electron microscopy (1, 34). Using a 5-nm-gold-labeled anti-VP1 antibody, the distribution of VP1 protein in the thin sections of the cells expressing this protein was determined (Fig. 4). There was abundant gold labeling throughout the cytoplasm, demonstrating the presence of unassembled VP1. In contrast, nuclear labeling was predominantly confined to the capsidlike structures. Control Sf9 cells, with or without AcNPV, were not labeled with the anti-VP1 antibody. Thus, cells infected with AcNPV-VP1 produce VP1 in the cytoplasm, and this protein is transported to the nucleus where it assembles into capsidlike particles of apparently uniform size.

Ionomycin treatment of Sf9 cells expressing VP1 causes cytoplasmic capsid assembly. To test whether increasing available cytoplasmic calcium might affect assembly of VP1 in the AcNPV-VP1-infected Sf9 cells, we added the calcium ionophore ionomycin to cells for 1 h at 72 h p.i., and the cells

FIG. 2. Phosphoamino acid analysis of VP1 labeled in vivo with $^{32}\text{P}_i$. (A) VP1 from polyomavirus-infected A31 mouse fibroblasts. (B) VP1 from AcNPV-VP1-infected Sf9 cells.

FIG. 4. Immunogold labeling of AcNPV-VP1-infected Sf9 cells. Cell at 72 h p.i. with 5-nm immunogold labeling of VP1. The immunoreactive cytoplasmic VP1 is not associated with recognizable particles, in contrast to the capsidlike particles in the nucleus (large arrow). n, nucleus; c, cytoplasm; nm, nuclear membrane. Bar = 0.2 μ m.

were then fixed, thin sectioned, and examined by electron microscopy. Although many cells contained abnormal vesicular structures and appeared necrotic, selected viable cells without nuclear or cytoplasmic membrane breakdown within the plane of the section were identified. As shown in Fig. 5, some of these cells contained clusters of intracytoplasmic capsidlike particles with a corrected diameter of 42.5 ± 6 nm (standard deviation) juxtaposed with those present in the nucleus. Although assembly may have been initiated by indirect effects of increased cytoplasmic calcium caused by ionomycin treatment, or by a toxic effect of this drug's action, these results are consistent with the hypothesis that intracellular calcium levels regulate assembly.

Purified capsidlike particles are relatively homogeneous and do not contain nucleic acid. Capsidlike particles were purified from infected Sf9 cells by the techniques previously developed for purification of viral nucleoprotein complexes from polyomavirus-infected cells (22). Electron microscopic examination of negatively stained preparations (Fig. 6) showed capsidlike particles that resembled the empty shells produced by self-assembly of purified, bacterially expressed VP1 at physiological salt concentrations with added calcium (24, 42). Particles of 46-nm approximate diameter predominate, which correspond to the normal $T = 7$ capsid size, but smaller isometric particles and occasional larger aggregates were also observed. Variability in particle size and shape may have resulted from self-assembly of VP1 released from the cytoplasm into the calcium-containing buffer used in the isolation procedure. From the micrographs, particles assembled in the nucleus cannot be distinguished from those that

may have formed during the isolation. All the capsidlike particles appear to be empty, which indicates that particles formed in the nucleus do not contain nucleic acid.

DISCUSSION

Polymorphism of the capsidlike aggregates formed by self-assembly of purified recombinant VP1 protein under various conditions *in vitro* suggested that additional control mechanisms are required to efficiently produce the $T = 7$ viral capsid *in vivo* (41, 42). Furthermore, specific interactions with the minor proteins VP2 and VP3 and the viral minichromosome must be involved in assembly of the complete virion. The intranuclear assembly of capsid structures in the AcNPV-VP1-infected Sf9 cells demonstrates that VP1 nuclear transport and capsid assembly may occur *in vivo* in the absence of other viral proteins. In addition, the particles in the infected Sf9 cells often appeared in regular arrays. Purification of the particles from the infected cells demonstrated their regular structure and established that most particles have the $T = 7$ morphology. Thus, although *in vitro* assembly of VP1 can be experimentally driven into a number of non- $T = 7$ assemblies by altering the pH or ionic strength of the association buffer (42), under physiologic conditions the bonding between capsomeres appears to be optimized for the $T = 7$ structure.

The particles formed in the AcNPV-VP1-infected Sf9 cells did not contain nucleic acid. VP1 binds to DNA in a sequence-nonspecific manner, with a K_d of $\approx 1 \times 10^{-11}$ M (35). Furthermore, DNA inhibits the *in vitro* assembly of

FIG. 3. Electron micrographs of Sf9 cells infected with AcNPV-VP1. Infected cell monolayers were prepared for microscopy as described in Materials and Methods. (A) Cell 72 h p.i. with arrow indicating capsidlike particles in the nucleus only, without cytoplasmic particles of similar morphology. (B) Separate field at higher magnification demonstrating uniformity of particle size and clustering near the nuclear membrane. n, nucleus; nm, nuclear membrane; c, cytoplasm; pm, plasma membrane; mt, microtubules. Bar = 0.2 μ m.

FIG. 5. Ionomycin-treated AcNPV-VP1-infected Sf9 cell. Electron micrograph of a cell 72 h p.i. treated with 5 μ M ionomycin for 1 h before fixation, with arrows indicating capsidlike particles in both the nucleus and the cytoplasm. n, nucleus; mt, microtubule, nm, nuclear membrane; pm, plasma membrane; vs, vesicular structure. Bar = 0.2 μ m.

VP1 into capsids, instead forming linear arrays of VP1 capsomeres aligned along the DNA (35). This result again suggests that additional factors, such as the minor capsid proteins VP2 and VP3, are involved in directing the assembly of VP1 capsomeres on the viral minichromosome. Relative underphosphorylation of VP1 on threonine did not appear to prevent capsid formation either in vitro or in Sf9 cells. Underphosphorylation of VP1 on threonine is associated with inefficient minichromosome encapsidation in *hr-t* mutant virus-infected mouse cells (23) and therefore appears to be functionally associated with packaging rather than with determination of the final capsid structure.

It is striking that particle assembly is exclusively intranuclear despite abundant immunoreactive cytoplasmic VP1.

Overexpression of VP1 by itself does not appear sufficient to drive particle assembly since the amount of cytoplasmic VP1 approximates that found in the nucleus, as determined by immunogold staining. Several observations suggest that calcium regulates assembly: (i) at physiological pH and ionic strength, the assembly of recombinant VP1 in vitro is driven by calcium (24, 41, 42); (ii) calcium chelation is required for efficient nondenaturing dissociation of virions (8, 13, 19); and (iii) X-ray crystallographic data suggest that calcium binding is involved in linking simian virus 40 pentamers (28a), as seen in the linkage of capsid proteins in other icosahedral viruses, e.g., tomato bushy stunt virus (26). The assembly of cytoplasmic VP1 after ionomycin treatment of AcNPV-VP1-infected Sf9 cells also suggests that calcium is important for

FIG. 6. Purified VP1 capsid preparations. (A) Negatively stained particles purified from Sf9 cells infected with AcNPV-VP1. (B) Normal $T = 7$ icosahedral capsids purified from polyomavirus-infected mouse kidney cells (stacked disk aggregate of tobacco mosaic virus protein added to calibrate magnification, which is the same as in panel A). (C) Gallery of selected, undistorted, negatively stained particles from AcNPV-VP1-infected cells. Those in the top row and the two larger particles in the second row correspond to the normal $T = 7$ icosahedral capsid. (D) Montage of frozen hydrated particles, most of which are the normal capsid size as calibrated by the tobacco mosaic virus stacked disk aggregate. The particle at the upper right corner is viewed nearly along a threefold axis of the icosahedral capsid. (Magnification is the same in panels C and D). Scale bars in panels B and D = 100 nm.

controlling capsid assembly. It is possible, however, that the effect of the drug on VP1 assembly is separate from its ability to increase the concentration of cytosolic calcium. For example, a protein promoting assembly might be activated by the increased calcium, or leakage of assembled particles from the nucleus to the cytoplasm could be induced by a toxic effect of the drug on the nuclear membrane. Nonetheless, it is tempting to speculate that the intranuclear assembly of capsids may reflect a differential in the available calcium between the cytoplasm and nucleus.

Cytosolic calcium concentrations and fluxes have been extensively investigated, and when nuclear and cytosolic calcium levels have been measured in the same cells, they are distinct, suggesting that the levels are independently regulated (12, 49, 50). Free calcium is approximately 3 orders of magnitude less than total calcium in each compartment. Most of the total cytosolic calcium is in a reservoir within the endoplasmic reticulum, but it is unknown how nuclear calcium is buffered. Although the total cytoplasmic calcium is shielded from interacting with proteins by its compartmentalization, the total nuclear calcium may not be spatially sequestered but available for those proteins whose avidity for calcium binding is greater than that of the nuclear buffer. Nuclear assembly of polyomavirus capsids may be an indicator that the nucleus and cytoplasm differ in available calcium and suggests that other proteins also use this mechanism to activate functions differentially in these subcellular compartments.

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REFERENCES

1. Banfield, W. G., C. J. Dawe, and D. C. Brindley. 1959. Intracellular and extracellular particles in tissue cultures inoculated with parotid-tumor agent (polyoma virus). *J. Natl. Cancer Inst.* 23:1123-1135.
2. Barkan, A., and J. E. Mertz. 1981. DNA sequence analysis of simian virus 40 mutants with deletions mapping in the leader region of the late mRNAs: mutants with deletions similar in size and position exhibit varied phenotypes. *J. Virol.* 37:730-737.
3. Barkan, A., R. C. Welch, and J. E. Mertz. 1987. Missense mutations in the VP1 gene of simian virus 40 that compensate for defects caused by deletions in the viral agnogene. *J. Virol.* 61:3190-3198.
4. Baumgartner, I., C. Kuhn, and E. Fanning. 1979. Identification and characterization of fast-sedimenting SV40 nucleoprotein complexes. *Virology* 96:54-63.
5. Benjamin, T. L. 1970. Host-range mutants of polyoma virus. *Proc. Natl. Acad. Sci. USA* 67:394-399.
6. Bina, M., V. Blasquez, S.-C. Ng, and S. Beecher. 1983. SV40 morphogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 47: 565-569.
7. Blasquez, V., S. Beecher, and M. Bina. 1982. Simian virus 40 morphogenetic pathway: an analysis of assembly defective tsB201 DNA-protein complexes. *J. Biol. Chem.* 258:8477-8484.
8. Brady, J. N., V. D. Winston, and R. A. Consigli. 1977. Dissociation of polyomavirus by the chelation of calcium ions found associated with purified virions. *J. Virol.* 23:717-724.
9. Brown, M., and P. Faulkner. 1977. A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *J. Gen. Virol.* 36:361-364.

10. Carswell, S., and J. C. Alwine. 1986. Simian virus 40 agnoprotein facilitates perinuclear-nuclear localization of VP1, the major capsid protein. *J. Virol.* **60**:1055–1061.
11. Caspar, D. L. D., and A. Klug. 1962. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* **27**:1–24.
12. Chandra, S., D. Gross, Y.-C. Ling, and G. H. Morrison. 1989. Quantitative imaging of free and total intracellular calcium in cultured cells. *Proc. Natl. Acad. Sci. USA* **86**:1870–1874.
13. Christiansen, G., T. Landers, J. Griffith, and P. Berg. 1977. Characterization of components released by alkali disruption of simian virus 40. *J. Virol.* **21**:1079–1084.
14. de Agostini, A. I., S. C. Watkins, H. S. Slayter, H. Youssoufian, and R. D. Rosenberg. 1990. Localization of anticoagulant active heparan sulfate proteoglycans in vascular endothelium: antithrombin binding on cultured endothelial cells and perfused rat aorta. *J. Cell Biol.* **111**:1293–1304.
15. Dubochet, J., M. Adrian, P. Schultz, and P. Oudet. 1986. Cryoelectron microscopy of vitrified SV40 minichromosomes: the liquid drop model. *EMBO J.* **5**:519–528.
16. Fernandez-Munoz, R., M. Coca-Prados, and M.-T. Hsu. 1979. Intracellular forms of simian virus 40 nucleoprotein complexes. I. Methods of isolation and characterization in CV-1 cells. *J. Virol.* **29**:612–623.
17. French, T. J., J. J. A. Marshall, and P. Roy. 1990. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. *J. Virol.* **64**:5695–5700.
18. French, T. J., and P. Roy. 1990. Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. *J. Virol.* **64**:1530–1536.
19. Friedmann, T., and D. David. 1972. Structural role of polyoma virus proteins. *J. Virol.* **10**:776–782.
20. Garber, E. A., M. M. Seidman, and A. J. Levine. 1978. The detection and characterization of multiple forms of SV40 nucleoprotein complexes. *Virology* **90**:305–316.
- 20a. Garcea, R. L. Unpublished data.
21. Garcea, R. L., and T. L. Benjamin. 1983. Host-range transforming gene of polyoma virus plays a role in virus assembly. *Proc. Natl. Acad. Sci. USA* **80**:3613–3617.
22. Garcea, R. L., and T. L. Benjamin. 1983. Isolation and characterization of polyoma nucleoprotein complexes. *Virology* **130**:65–75.
23. Garcea, R. L., K. Hofer, and T. L. Benjamin. 1985. Virion assembly defect of polyomavirus hr-t mutants: underphosphorylation of major capsid protein VP1 before viral DNA encapsidation. *J. Virol.* **54**:311–316.
24. Garcea, R. L., D. M. Salunke, and D. L. D. Caspar. 1987. Site-directed mutation affecting polyomavirus self-assembly in vitro. *Nature (London)* **329**:86–87.
25. Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. DeWilde. 1989. Assembly and release of HIV-1 precursor pr55^{gag} virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**:103–112.
26. Hogle, J., T. Kirchhausen, and S. C. Harrison. 1983. Divalent cation sites in tomato bushy stunt virus: difference maps at 2.9 Å resolution. *J. Mol. Biol.* **171**:95–100.
27. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
28. Leavitt, A. D., T. M. Roberts, and R. L. Garcea. 1985. Polyoma virus major capsid protein, VP1: purification after high level expression in *Escherichia coli*. *J. Biol. Chem.* **260**:12803–12809.
- 28a. Liddington, R. Personal communication.
- 28b. Liddington, R., and S. C. Harrison. Unpublished data.
29. Loudon, P. T., and P. Roy. 1991. Assembly of five bluetongue virus proteins expressed by recombinant baculoviruses: inclusion of the largest protein VP1 in the core and virus-like particles. *Virology* **180**:798–802.
30. Luckow, V. A., and M. D. Summers. 1989. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. *Virology* **170**:31–39.
31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. Margolskee, R. F., and D. Nathans. 1983. Suppression of a VP1 mutant of simian virus 40 by missense mutations in serine codons of the viral agnogene. *J. Virol.* **48**:405–409.
33. Matsudaira, P. T. 1989. A practical guide to protein and peptide purification for microsequencing. Academic Press, Inc., San Diego, Calif.
34. Mattern, C. F. T., K. K. Takemoto, and W. A. Daniel. 1966. Replication of polyoma virus in mouse embryo cells: electron microscopic observation. *Virology* **30**:242–256.
35. Moreland, R. B., L. Montross, and R. L. Garcea. 1991. Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1. *J. Virol.* **65**:1168–1176.
36. Ng, S. C., J. E. Mertz, S. Sanden-Will, and M. Bina. 1985. Simian virus 40 maturation in cells harboring mutants deleted in the agnogene. *J. Biol. Chem.* **260**:1127–1132.
37. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007–4021.
38. Rasmussen, L., J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda. 1990. Characterization of virus-like particles produced by a recombinant baculovirus containing the gag gene of the bovine immunodeficiency-like virus. *Virology* **178**:435–451.
39. Rayment, I., T. S. Baker, D. L. D. Caspar, and W. T. Murakami. 1982. Polyoma virus capsid structure at 22.5 Å resolution. *Nature (London)* **295**:110–115.
40. Resnick, J., and T. Shenk. 1986. Simian virus 40 agnoprotein facilitates normal nuclear location of the major capsid polypeptide and cell-to-cell spread of virus. *J. Virol.* **60**:1098–1106.
41. Salunke, D. M., D. L. D. Caspar, and R. L. Garcea. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**:895–904.
42. Salunke, D. M., D. L. D. Caspar, and R. L. Garcea. 1989. Polymorphism in the assembly of polyomavirus capsid protein VP1. *Biophys. J.* **56**:887–900.
43. Shenk, T. E., J. Carbon, and P. Berg. 1976. Construction and analysis of viable deletion mutants of simian virus 40. *J. Virol.* **18**:664–671.
44. Summers, M. D., and G. E. Smith. 1986. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555. Texas Agricultural Experiment Station, College Station, Tex.
45. Tooze, J. 1980. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
47. Urakawa, T., M. Ferguson, P. D. Minor, J. Cooper, M. Sullivan, J. W. Almond, and D. H. L. Bishop. 1989. Synthesis of immunogenic, but non-infectious, poliovirus particles in insect cells by a baculovirus expression vector. *J. Gen. Virol.* **70**:1453–1463.
48. Watkins, S. C., J. L. Samuel, F. Marotte, B. Bertier-Savalle, and L. Rappaport. 1987. Microtubules and desmin filaments during onset of heart hypertrophy in rat: a double immunoelectron microscope study. *Circ. Res.* **60**:327–336.
49. Williams, D. A., P. L. Becker, and F. S. Fay. 1987. Regional changes in calcium underlying contraction of single smooth muscle cells. *Science* **235**:1644–1648.
50. Williams, D. A., K. E. Fogarty, R. Y. Tsien, and F. S. Fay. 1985. Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2. *Nature (London)* **318**:558–561.