Cytoplasmic Structures Associated with an Arbovirus Infection: Loci of Viral Ribonucleic Acid Synthesis

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Unique cytoplasmic structures, herein designated as type I cytopathic vacuoles (CPV-I), are found in chick embryo cells early in the logarithmic phase of Semliki Forest virus replication. High resolution autoradiography demonstrated that the CPV-I are loci of ^3H -uridine incorporation. This evidence correlates well with previous biochemical data and electron microscopy of the subcellular fractions active in Semliki Forest virus ribonucleic acid synthesis. Origin of the CPV-I within host cell cytoplasm is confirmed by the distribution of electron-dense tracer particles and sequential ultrastructural observations.

The formation of unique intracytoplasmic vacuoles within cells infected by Semliki Forest virus (SFV) and related members of the arbovirus group has been demonstrated by electron microscopy in both cell cultures and the cerebral tissues of experimental animals (21, 24, 32). The cytopathic vacuoles (CPV) may be subdivided into two morphologically distinct groups. (i) A type of CPV recently recognized in chick embryo cells infected by SFV (1, 14) or Sindbis virus (*unpublished data*). These occur relatively early in the infectious cycle and are, therefore, designated CPV-I. They are characterized by a regular series of membrane buds or spherules which are attached to the interior surface of the vacuoles. The buds or spherules are formed of membranes with unit structure (1), and may contain an electron-dense central spot. (ii) The type of CPV that was originally described in cells infected by Western equine encephalitis virus (23). These vacuoles are encircled on the cytoplasmic aspect by closely spaced particles of uniform size (25 to 30 nm). They are most numerous in the late (linear) phase of the virus replication cycle and are designated CPV-II. CPV-II have been observed in cell systems infected by SFV and several other group A arboviruses (3, 10, 23-26).

Preliminary studies in this laboratory showed that CPV-I arise during the early stages of SFV infection and are present in the cytoplasmic fractions which incorporate ^3H -uridine (14). In SFV infection, the new viral ribonucleic acid (RNA) is closely associated with cytomembranes and may be released from the membrane sediment by

sodium deoxycholate (12, 14). We demonstrated the participation of CPV-I in viral RNA synthesis by means of high resolution autoradiography. Ultrastructural studies of the infected cells at sequential intervals and the distribution of an electron-dense tracer have provided additional information concerning the origin and fate of the arbovirus CPV.

MATERIALS AND METHODS

Virus and cell cultures. The Kumba strain of Semliki Forest virus was grown at 37 C in primary cultures of chick embryo cells (15). Monolayers of chick embryo fibroblasts were infected at virus-cell multiplicities of 20:1 to 500:1. Virus was titrated by plaque assay (16). In some experiments, virus was absorbed to the cells overnight at 4 C, either in medium containing 1 μg of actinomycin D per ml or in medium without actinomycin D. In other experiments, virus was added directly to the monolayers, with or without actinomycin D, and maintained at 37 C. Cultures were studied at selected intervals (0.5 to 16 hr) after the initiation of infection. In cold (4 C) virus absorption, the period of infection was considered to begin from the moment of rewarming to 37 C (13, 15). Eight separate experiments were conducted to study the ultrastructural sequence of virus development at relatively low multiplicity of infection (20:1 to 40:1). In addition, two experiments employing a high virus multiplicity (500:1) were conducted to trace virus entry and to detect early evidence of replication (up to 3 hr). The preparation of high titer SFV pools for experiments requiring a high virus to cell multiplicity has been reported previously (13).

Electron microscopic preparations. Cell monolayers were scraped from the petri dish with a rubber police-

man and transferred to centrifuge tubes along with the original culture fluid. Some of the supernatant fluid was withdrawn before centrifuging at $785 \times g$ for 5 min. The resulting pellet was fixed for 1 to 2 hr with 3% glutaraldehyde (4) in 0.1 M phosphate buffer (pH 7.3). In experiments employing Thorotrast (thorium dioxide) or ^3H -uridine, cell monolayers were fixed directly in the petri dishes before centrifugation. Glutaraldehyde-fixed pellets were washed in 0.1 M phosphate buffer containing sucrose, prior to 1 hr of postfixation in 1% osmium tetroxide which was also buffered with 0.1 M phosphate. After rapid dehydration in graded 70 to 100% ethyl alcohol solutions, the pellets were embedded in Luft's Epon formula (14). Sections were cut by an automatic ultratome with diamond and glass knives. They were mounted on uncoated copper mesh grids, stained with 10% uranyl acetate in methanol, and examined at original magnifications up to 18,000 in an RCA 3F electron microscope operated at 50 kv.

Pulse labeling. At indicated times after virus infection, the culture medium was decanted, and 1 ml of Eagle's medium containing 100 μC of ^3H -uridine (20 c/mm) was added for 3 min. The monolayers were then quickly washed five times with chilled 0.85% NaCl (containing uridine) and fixed immediately with glutaraldehyde. In control tests, monolayers were fixed with Carnoy's ethyl alcohol-ether fluid, and acid-precipitable radioactivity was counted with a Packard Tri-Carb scintillation spectrometer model 3003 (Packard Instrument Co., Downers Grove, Ill.).

Autoradiography. Thick epoxy sections (0.5 to 1.0 μm) for light microscopy were picked up on 'subbed' slides (2) and air-dried. They were coated with Kodak AR-10 stripping film (27) or Ilford L-4 Nuclear Research Emulsion [Ilford, Ltd., Ilford, Essex, England (2)]. The procedures for electron-microscopy were based on the method of Caro et al. (2). Control grids without sections were covered with emulsion, exposed to room light, and examined in the electron microscope with or without development to check grain distribution. Other control grids with unlabeled sections or without any sections were coated, stored for 2 to 10 weeks, and developed to determine background grains. Thick (0.5 to 1 μm) sections were stained through the emulsion with 0.1% Toluidine Blue in aqueous solution. Thin sections were examined in the electron microscope without staining.

Thorotrast tracer. In four groups of experiments, a colloidal suspension of 24 to 26% Thorotrast was either added to cell pellets during fixation or introduced into the culture medium (1 to 2 cc/10 cc of medium) for 5 to 60 min preceding fixation (no tracer added). For the tracer-fixation procedure, some cell pellets were prefixed in 3% glutaraldehyde (4) for 5 min before the addition of 3% glutaraldehyde containing 1 to 2:10 parts of the volume of the 24 to 26% Thorotrast. Other pellets were fixed directly in the Thorotrast-glutaraldehyde mixture. A similar volumetric proportion of the 24 to 26% Thorotrast was maintained in the phosphate buffer-sucrose rinse and in the 1% osmium tetroxide postfixation.

RESULTS

Virus growth characteristics. In the chick embryo cells treated with actinomycin D and incubated at 37 C with 20 plaque-forming units (PFU) of SFV per cell, the titers of virus infectious units increased exponentially during a period of 1 to 5 hr (Fig. 1). After 8 hr, production of infectious virus reached a plateau. The rising titer of cell-associated virus closely paralleled the titration curve of virus in the culture fluid, both in slope and in maximal height.

Uninfected cells. Chick embryo cells in primary culture displayed a considerable pleomorphism of cytological appearance, but a majority resembled fibroblasts or histiocytes. Most cells contained an abundance of dispersed ribosomes in the cytoplasmic matrix. Aggregation of ribosomes into crystalloid arrays was occasionally noted within the cytoplasmic matrix or adjoining the limiting membranes of endoplasmic reticulum. Cytoplasmic lipid droplets, phagocytic vacuoles, and fenestrations were common. The latter often measured more than 2 μm in diameter. In cells treated with actinomycin D, the nucleolar changes were characteristic (29), and cytoplasmic vacuoles containing autophagic whorls of membrane remnants frequently appeared (9). Neither in treated nor untreated control cells, however, were the arbovirus CPV ever identified.

FIG. 1. Titers of SFV in chick embryo cells at various times after infection with a multiplicity of 20 plaque-forming units per cell.

Infected cells. In six different series of experiments, the chick cells were examined after infection with 20 to 50 PFU of SFV per cell. The earliest ultrastructural changes attributable to SFV were the occurrence of surface budding virus particles and CPV-I at 2.5 to 3 hr. By 3 to 4 hr after the initiation of infection, cross sections through 10 to 20% of cells observed in random planes disclosed CPV-I (Fig. 2-6). Virus nucleoids budding through the plasmalemma to form mature virions were often present in the same cells as the CPV-I (Fig. 3) or in planes of section lacking the vacuoles. Empty membrane projections were often observed adjacent to or in regions of virus budding (Fig. 2, 3). Cells containing CPV-II (Fig. 7) were noted after 4 hr of SFV infection. In the late stages of infection (8 to 16 hr), a few "mixed" cytopathic vacuoles (CPV-III) combined morphological features both of CPV-I and of CPV-II (Fig. 8).

Typical CPV-I were distinguished by their relatively large diameter (0.6 to 2 μm) and the regularly spaced membranous spherules that projected from their interior surfaces (Fig. 2-6). The CPV-I appeared to originate from compact clusters of 50-nm spherules which were observed in the cytoplasmic matrix (Fig. 4-6). Rarefaction of cytoplasm within the clusters apparently produced a central vacuolar space. Mature CPV-I were clearly defined by a limiting membrane (Fig. 4), and the characteristic inner spherules often displayed a dense central spot (Fig. 5). The inner membranous projections of CPV-I were not always uniform and circular in profile. In some sections, they appeared digital or droplike and often resembled the empty membrane projections of similar size which appeared in aggregates on the plasmalemma of infected cells (Fig. 2). Membrane degeneration within CPV-I was a common feature, particularly as infection progressed (Fig. 7). An intimate relationship of CPV-I to the Golgi zone or rough endoplasmic reticulum was often noted (Fig. 5, 6).

The CPV-II appeared to arise from dilated regions of the endoplasmic reticulum (Fig. 9). As in other group A arbovirus infections, the CPV-II were surrounded by dense particles believed to represent viral nucleoids (23, 25). In planes tangential to the surface of CPV-II, the 30-nm nucleoids were occasionally arranged in compact crystalloid arrays without intrinsic symmetry. CPV-II were generally smaller in diameter than the CPV-I, averaging 0.35 μm . As the infection progressed, many CPV-II contained mature, membrane-enveloped virions within their lumens (Fig. 10), and complex intravacuolar herniations of the types described by Erlandson

et. al. (10) occurred in the latest stages examined (16 hr).

The cycle of virus development was asynchronous at all multiplicities of infection studied; nevertheless, certain trends in the development of cytopathic vacuoles were evident (Fig. 11). The CPV-I were most prominent during the early phase of infection. After 4 hr, the relative proportion of CPV-II increased rapidly, and the proportion of CPV-I observed in any plane of section of a particular cell appeared inversely related to the numbers of CPV-II. Some images suggested a degeneration of the membrane spherules lining CPV-I at about the same time as CPV-II appeared (Fig. 7). The CPV-III were not observed before 8 hr of infection and were never numerous.

Intracytoplasmic aggregates of ribosomes were occasionally noted during the course of SFV infection (Fig. 8). In some instances, these images probably represented tangential views along the surface of endoplasmic reticulum. A peculiar phenomenon in the terminal stages of infection was a proliferation of smooth endoplasmic reticulum in the host cell. This effect has also been noted in the late stages of some DNA virus infections (P. M. Grimley, *unpublished data*).

Autoradiography. In trial experiments, control cells incubated with ^3H -thymidine or pulse-labeled with ^3H -uridine displayed an appropriate and discrete localization of developed silver grains over the nucleus (Fig. 12) within periods of 2 to 10 weeks of emulsion exposure. Control cells treated with actinomycin D and labeled with ^3H -uridine in an identical fashion displayed no evidence of localized grain development after 10 weeks of exposure. Background grains were negligible (<1 per oil immersion field). Direct recording of acid-precipitable counts in the control, actinomycin D-treated, and actinomycin D-treated and virus-infected (20 PFU, 4 hr) cultures disclosed: (i) 639 counts per min per μg of protein, (ii) 27 counts per min per μg of protein, and (iii) 142 counts per min per μg of protein. Specific activity in the virus-infected cells at 4 hr was thus approximately five times that in cells treated with actinomycin D only.

In cells infected with 20 PFU of SFV for 6 hr and pulse-labeled for 3 min with ^3H -uridine, localized cytoplasmic grains were detected by light microscopy within 2 to 5 weeks of exposure. Approximately 30% of the cell sections were very heavily labeled after 5 weeks, and grains were generally concentrated near the cell periphery. Grains were rarely detected over nuclei or outside of cell sheets. Few grains appeared over the identically processed, actinomycin D-treated

FIG. 2. CPV-I in a cell infected with SFV for 2.5 hr. Profiles of 50-nm membrane projections within Ia are uniformly circular. Projections in Ib are more elongate and resemble irregular protrusions of the adjacent plasmalemma (p). $\times 24,600$.

FIG. 3. CPV-I in close proximity to plasmalemma. Mature virus particles (V) are budding in close proximity to hollow plasma membrane protrusions (p). $\times 24,600$.

FIG. 4. Group of CPV-I in cell infected for 5 hr. Clusters of cytoplasmic spherules (arrows) appear to represent an early stage in the vacuole formation. Flocculent material within mature CPV-I may represent rarefied remnants of the original cytoplasmic matrix. $\times 37,000$.

FIG. 5. CPV-I in vicinity of Golgi apparatus (G). Many of the membrane spherules appear to have detached from Ic, whereas those in Ia are regularly arranged and intact. $\times 26,200$.

FIG. 6. Close proximity of CPV-I to flat cisternae of rough endoplasmic reticulum (ER). Degenerated membrane whorl within Ia. Arrow indicates early form of CPV-I. $\times 26,400$.

FIG. 7. Cell infected for 5 hr. Degenerating vacuole (Ic) presumably derived from CPV-I (Fig. 11). CPV-II are considerably smaller in diameter (II). $\times 26,400$.

FIG. 8. Mixed vacuole (CPV-III). Inner membrane projections resemble CPV-Ib (Fig. 11), but surrounding dense particles resemble nucleoids of CPV-II. Note crystalloid aggregation of ribosomes on adjacent endoplasmic reticulum (ER). $\times 26,400$.

control sections examined 5 to 14 weeks after exposure. In sections examined by electron microscopy, discrete grain development could be detected over infected cells within 4 weeks of exposure. Background grains at this time were negligible. In some cell cross sections, the cytoplasmic labeling appeared random; however, in

many instances, a significant number of the cytoplasmic grains were concentrated over CPV-I, often in sections in which few or no grains developed over other portions of the cell (Fig. 13). Since the CPV-I were obscure in the unstained emulsion-coated sections examined by electron microscopy, until after development of the

FIG. 9. Typical CPV-II in cell infected for 4 hr. Note close relationship of some vacuoles (IIa, IIb) to cisternae of rough endoplasmic reticulum (ER) or perinuclear cisternum (PN). Tangential plane of sections shows close packing of nucleoids (arrow) investing CPV-II. $\times 55,400$.

FIG. 10. CPV-II containing mature virus particles within lumen. Cluster of particles at cell surface (arrow) may have been released from a similar vacuole in process of "exocytosis." $\times 52,800$.

FIG. 11. Schematic diagram based upon electron micrographs and suggesting possible transitions among CPV. CPV-Ia with typical membrane spherules appear to arise from spherular clusters in cytoplasmic matrix. CPV-Ib with elongate membrane projections may represent a secondary alteration. A relationship of this structure to surface protrusions from the plasmalemma is suggested by morphology and Thorotrast tracer experiments. Degeneration to CPV-Ic could be initiated by hydrolytic enzymes from the Golgi region. Nucleoids surrounding CPV-IIa may mature by budding through plasmalemma or limiting membrane of vacuole (CPV-IIb). Exit of mature virions in transport vacuoles (CPV-III) is suggested by Thorotrast tracer experiments and by the relatively high proportion of cell-associated virus (Fig. 1).

negative plates, subjective bias in field selection on the fluorescent screen was largely prevented. In a majority of the cell sections labeled by tritium, the largest proportion of grains were concentrated near the cell surface (Fig. 13). This phenomenon was not observed in identically washed, actinomycin D-treated or untreated controls incubated in the same concentrations of ^3H -uridine.

Thorotrast experiments. The electron-dense tracer particles of Thorotrast were present in sufficient concentration to outline the surface of more than 25% of cell cross sections examined. In pellets of control chick embryo cells, fixed prior to introduction of Thorotrast or fixed in a Thorotrast mixture, electron-dense tracer particles were frequently identified within large cytoplasmic fenestrae or surface-connected recesses. Similar findings were the rule after 6 hr of SFV infection. Tracer particles were never identified in CPV, even though closely proximate fenestrae or digestive vacuoles contained them (Fig. 14). Occasionally, subplasmalemmal ves-

icles containing mature virions also disclosed a few tracer particles.

When the Thorotrast was added to the tissue culture media before cell fixation, results paralleled those described above during the first 15 to 45 min of incubation. By 1 hr, however, rare CPV-Ib (Fig. 11) contained electron-dense particles. Tracer particles were never detected in CPV-II. Significantly, vacuoles located just beneath the cell surface and containing mature virions (CPV-IIc) were usually free of tracer particles even in cells heavily outlined by the Thorotrast.

High multiplicity infections. With purified and concentrated virus preparations (15), mature virions with cell detritus from the heavy inoculum were identified at cell surfaces for up to 1 hr. Under these conditions, many particles entered the host cells by phagocytosis, sometimes in the vicinity of surface "pits" (Fig. 15). Phagocytic vacuoles containing complete virions or empty virus envelopes persisted within many cells for as late as 2 hr after inoculation (Fig. 16). Actual

penetration of individual virus particles, from phagocytic vacuoles into the cytoplasmic compartment, could not be detected. In representative culture samples examined at 1 and 2 hr

after high titer inoculation, CPV were not yet present; however, in some cells, condensation of ribosomes along the rough endoplasmic reticulum was unusually prominent (Fig. 17). As in the

FIG. 12. Autoradiograph of cell from chick embryo culture pulse labeled with ^3H -uridine. Grain development localized over nucleus (N). $\times 11,000$.

FIG. 13. Autoradiographs of actinomycin D-treated cells infected for 6 hr with SFV and pulse labeled with ^3H -uridine. A. Grains concentrated near plasmalemma. $\times 13,500$. B. Grains concentrated in region of CPV-I. $\times 25,870$.

FIG. 14. Section of cell from infected culture, incubated with colloidal thorium dioxide for 1 hr. Electron-dense tracer has entered a small subplasmalemmal vacuole (arrow), but it is not observed within CPV-I. $\times 32,000$.

FIG. 15. Clusters of mature SFV virions at surface of cell 30 min after high multiplicity infection. Note proximity to coated "pits" (arrows) and filopodial extension of cytoplasm (F). $\times 39,400$.

FIG. 16. Clusters of mature SFV virions within cytoplasmic vacuoles at 30 min after high multiplicity infection. $\times 39,600$.

FIG. 17. Crystalloid condensation of ribosomes in cell infected for 2 hr (high multiplicity). Note proximity of endoplasmic reticulum (ER) to plasma membrane. $\times 42,200$.

FIG. 18. Cell infected with high multiplicity of SFV. CPV-II are well developed at 3 hr after infection, and some contain mature virions (IIb, IIc). These contrast with degenerating membranous spherules (Ic) in CPV-I. $\times 42,600$.

low multiplicity infections, well-developed CPV-I appeared at about 3 hr (Fig. 18). These occurred only in a small fraction of cell cross sections. CPV-I and CPV-II were often present simultaneously (Fig. 18). Some CPV-II contained mature virions, and surface budding virus was also evident at this time. Digital membrane protrusions with hollow cores were occasionally observed in rows along the surface of infected cells.

DISCUSSION

Membrane association of viral RNA. Recent studies of chick embryo cells infected with SFV have suggested that replication of both viral RNA and protein occurs in association with cytomembranes (12-14). The replicative intermediate (12) and the RNA polymerase (20) of SFV have been recovered with membrane fractions of the host cell cytoplasm. Up to 75% of the viral RNA produced early in infection may be released from the cytomembrane fraction by simple treatment with deoxycholate (1, 2). CPV-I are present in cytoplasmic fractions containing the early forms of viral RNA (14), and autoradiographic and electron microscopic observations now suggest that they may serve as important sites for SFV RNA synthesis. The distribution of silver grains suggests that the CPV-I limiting membrane, rather than the vacuole interior, is the actual site of specific ^3H -uridine uptake (6). Heavy grain localization at the cell surfaces demonstrates that the plasmalemma may also provide a site for viral RNA replication. The relative contributions of cell surface and intracytoplasmic loci of SFV-RNA synthesis will be difficult to assess until more refined differential separation of cytomembrane sediments is feasible. Concentration of new viral RNA in discrete cytoplasmic loci or "factories" finds precedent in both RNA and deoxyribonucleic acid virus infections (7, 19, 28).

Significance of CPV in arbovirus development. Our observations of CPV-I as early as 2 to 3 hr after high- or low-titer virus inoculations and CPV-II within 3 to 4 hr refute an earlier contention (1) that CPV are unrelated to the phase of effective virus multiplication. We also observed CPV-I in the early logarithmic phase of Sindbis virus replication (*unpublished data*). Careful reexamination of other cell systems during the log phase of arbovirus growth may disclose close morphological as well as biochemical parallels (30) to the events described in SFV infection. The "globoid bodies" described in nerve cells infected by Japanese B encephalitis bear some resemblance to the CPV-I of SFV (34).

We initially considered the possibility that CPV-I might represent cytoplasmic depots related to virus entry. A remarkably similar vacuole occurs within 15 min of high multiplicity polyoma infection (11), and structures reminiscent of arbovirus CPV-II may arise by "pinocytotic activity" (22) during the multiplication of mammary tumor agent. In SFV infection, CPV-I were never observed less than 2 hr after high- or low-titer infections; phagocytic vacuoles containing clumps of degenerating viruses could easily be distinguished during this period. The failure of the electron-dense tracer to penetrate CPV-Ia in several experiments helps to exclude an origin from invaginating surface membranes. The presence of Thorotrast in occasional CPV-Ib or CPV-Ic after 45 min of incubation indicates that the latter structures might communicate with surface membrane, perhaps in the zones displaying the unusual configuration of multiple vesicular projections (18). It is also conceivable that the Thorotrast observed in CPV-I after 45 min reflects secondary fusion of CPV-I with lysosomal bodies (17). This could explain the CPV-I degeneration and their proportionate decline in numbers with respect to CPV-II. A close association of CPV-I with the Golgi region has occasionally been noted (Fig. 5).

Formation of CPV-II and release of SFV. Several investigators have suggested previously that the 25- to 30-nm "nucleoids" surrounding arbovirus CPV-II are virus precursors (10, 23-25). These nucleoids evidently correspond to the 140S cell fraction isolated by Friedman (12, 14).

Precursor particles investing the CPV-II evidently acquire an external membrane coat in transit to the vacuole lumen (10, 23). The relatively higher titer of cell-associated virus obtained in our experiment and a close parallel to the rising curve of released virus lend strong support to the morphological evidence of mature virus formation in CPV-II. Since the findings of Cheng (5) indicate an exceptionally high ratio of infectious to visible virions in SFV infection, we may reasonably expect that most of the intravacuolar virions observed during the log growth phase are indeed biologically active. The absence of electron-dense tracer within many CPV-II (Fig. 11) after 45 min of incubation in Thorotrast medium indicates that mature virions observed in these vacuoles may be leaving rather than entering the infected cells.

Correlation of morphological and biochemical observations. Several forms of viral RNA are produced during SFV infection (12, 14, 15). A partially ribonuclease-resistant, polydisperse form containing a 16S "core" apparently is the replica-

tive intermediate (12). It is membrane-associated and more rapidly labeled than the other RNA forms. Infectious RNA sediments at 42S and is present in a 140S particle that also contains viral protein (13). Autoradiographic labeling with ^3H -uridine now localizes some of the early viral RNA to the plasma membrane region and CPV-I.

The bulk of protein synthesis in SFV-infected chick cells is also membrane-associated (13). A spatial proximity of virus assembly sites to membrane-bound host cell ribosomes thus might be anticipated. Our electron micrographs are consistent with some earlier impressions that CPV-II derive from the endoplasmic reticulum of infected cells (3, 25). The frequent aggregation of ribosomes observed in infected cells, particularly high multiplicity infections (Fig. 8, 17), may reflect virus-directed protein synthesis, since 95% of host-cell protein synthesis is inhibited by the actinomycin D pretreatment (13).

Migration of early viral RNA from CPV-I to CPV-II could plausibly occur prior to incorporation of the 42S infectious RNA form into the 140S "nucleoids" which provide the first morphological evidence of virus core formation. This postulated migration of early viral RNA would be compatible with the temporal sequence of ultrastructural events; CPV-I precede CPV-II in appearance and decline in relative numbers as infection progresses. Some micrographs (Fig. 6) suggest a close proximity of CPV-I to the rough endoplasmic reticulum; considering the mobility of the latter, frequent contacts must occur. Contact of the endoplasmic reticulum with the plasmalemma may occur with significant frequency (Fig. 17), and has also been observed in herpes virus infections (V. Bedoya et al., J. Natl. Cancer Inst., *in press*). Such a phenomenon may explain the frequent maturation of SFV virions near the cell surface. A spatial dissociation of RNA replicative and viral assembly sites also appears to occur in poliovirus infection (8).

Integrating the observations to date, a tentative sequence of events in the SFV reproductive cycle may be postulated. (i) The RNA replicative intermediate is formed on the plasma membrane or at intracellular loci (CPV-I). (ii) Assembly of infectious RNA within an RNA-protein core or nucleoid proceeds on the exterior of CPV-II, derived from endoplasmic reticulum or at the plasma membrane. (iii) Mature virions form from the 140S nucleoids as they traverse either the plasma membrane or CPV-II limiting membrane and obtain an external envelope.

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