

Interaction of Frog Virus-3 with the Cytoskeleton.

I. Altered Organization of Microtubules, Intermediate Filaments, and Microfilaments

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ABSTRACT The progressive cytoskeletal alterations of frog virus 3-infected baby hamster kidney (BHK) and fathead minnow (FHM) cells were studied by immunofluorescence and electron microscopy. The virus assembly sites, which contain viral genomes and viral proteins, were detected in the cytoplasm at 4 h (FHM) or 6 h (BHK) and mature virions appeared 2 h later. When infected cells were treated with Triton X-100, the assembly sites were found in association with the cytoskeleton. In infected cells, the number of microtubules progressively decreased but a few microtubules traversing in the vicinity of the assembly sites remained intact. Early in infection, the intermediate filaments retracted from the cell periphery, delimited the forming assembly sites, and remained there throughout infection. We suggest that intermediate filaments are involved in the formation of assembly sites. In addition, the filaments either by themselves or in conjunction with microtubules may anchor the assembly sites near the nucleus. The microfilament bundles (stress fibers) disappeared with the formation of assembly sites, and late in infection many projections containing microfilaments and virus particles appeared at the cell surface. The observation suggests a role for microfilaments in virus release. Taken together, these results provide the first example of a virus-infected cell in which all three cytoskeletal filaments show profound organizational changes and suggest an active participation of the host cytoskeleton in viral functions.

It is now well established that all eucaryotic cells contain a detergent-insoluble structural framework termed the cytoskeleton (5, 30, 38, 48). The major components of the cytoskeleton are three morphologically and chemically distinct filaments: microtubules, intermediate filaments, and microfilaments (for reviews, see references 4, 19, 29). The three filaments have been implicated in many cellular functions including the maintenance of cell shape, cell motility, cell adhesion, cell division, intracellular transport, organelle localization, and movement of chromosomes and organelles. In addition to the major filaments, an anastomosing network of finer filaments of unknown function and composition termed the "microtrabecular lattice" has also been identified in whole cells and cytoskeletons by high voltage electron microscopy (41, 49).

Upon infection, most viruses induce extensive morphological and functional alterations in the host cell. On the basis of our knowledge of the function of the normal cellular cytoskeleton, we postulate that some of these alterations are brought about by the modification of the host cytoskeleton for viral functions.

The work of Lenk and Penman (31) has shown an association of poliovirus replication complex with the cytoskeleton and gross morphological and biochemical alterations of the cytoskeleton in infected cells. These authors suggested that the changes observed in the cytoskeleton are not due to gratuitous virus-induced damage but are the result of an active modification of the normal cellular machinery by the virus. That a single virus protein could cause dramatic alterations in the organization of the cytoskeleton was demonstrated in studies with virus-transformed cells (1, 10, 23, 24, 42). Studies with poxvirus-infected cells have revealed an intimate association of virus assembly sites ("factories") with the cytoskeleton (21, 46). In these cells, the virus was also shown to induce new actin-containing surface projections ("specialized microvilli") for its release (20, 27). Significant changes were observed in the organization of microfilaments in cells infected with vesicular stomatitis virus and Newcastle disease virus (39), although their relationship to viral functions was not understood. Electron microscopic studies show the association of virions with micro-

tubules or with the spindle apparatus in cells infected with reovirus (6), adenovirus (8, 32), and barley stripe mosaic virus (35). The significance of these observations remains unclear, although it has been postulated (6, 8, 32, 35) that microtubules have a role in intracellular and cell-to-cell transport of virions.

We have begun detailed ultrastructural, immunocytochemical, and biochemical studies to examine the role of host cytoskeleton in viral functions in frog virus-3 (FV3) infected tissue cultures cells. There are several reasons for choosing this system for analysis. FV3 is a well-characterized icosahedral animal DNA virus belonging to iridovirus group (15). The genome (100×10^6 mol wt) lacks the cross-linked termini seen in poxvirus DNA and the inverted repeats observed in herpesvirus genome (37), but it is circularly permuted and terminally redundant as in certain bacteriophages (16, 37). Unlike other animal DNA viruses which replicate and assemble either in the nucleus (e.g., herpesviruses) or in the cytoplasm (e.g., poxviruses), FV3 uses the nucleus for its DNA and RNA synthesis, but assembles in the cytoplasm (14, 17). FV3, like poxvirus (7), forms distinct, morphologically recognizable assembly sites within the cytoplasm early in infection (9). The viral genomes and viral proteins synthesized elsewhere accumulate at these sites for virus assembly (14). The virus particles then move from these sites to the plasma membrane for budding (9). Thus, the FV3-infected cell provides an ideal system to examine the possible role of the cytoskeleton, in the transport of viral components to the assembly site, in the formation of assembly sites, and in the release of finished virions.

We present results of immunofluorescence and electron microscopic studies of FV3-infected BHK (baby hamster kidney) and FHM (fathead minnow) cells. We describe the progressive alterations in the organization of microtubules, intermediate filaments, and microfilaments in the infected cells, structure of the assembly site and the association of the assembly site with the cytoskeleton. The results suggest an active participation of the host cytoskeleton in viral functions.

MATERIALS AND METHODS

Cells and Viruses: BHK and FHM cells were grown on 20-mm² glass coverslips in Eagle's medium supplemented with 5% fetal calf serum. The cells were infected with FV3 at a multiplicity of 25–30 plaque forming units/cell as described (14). Sparse cultures were used in immunofluorescence and confluent monolayers were used in electron microscopy.

Cytoskeleton Preparations: The cytoskeletons were prepared exactly as described by Schliwa and van Blerkom (40).

Antibodies: Antisera against FV3 antigens were obtained by multiple inoculation of rabbits with whole virus (33). Monoclonal antibodies against an FV3 structural protein were obtained from hybridoma cell lines as described (26). Affinity-purified sheep antibody to bovine brain tubulin was purchased from Caabco (Houston, TX). The anti-58,000-dalton antiserum was provided by Dr. R. Hynes (Hynes and Destree, reference 22) and the rhodamine (RITC)-conjugated-phalloidin (for microfilament localization) was obtained from Drs. R. Pollack, D. Alcorta (both of Columbia University), and T. Weiland (Max Planck Institute). The fluorescein (FITC)-conjugated antibodies (goat anti-rabbit IgGs, rabbit anti-mouse IgGs, rabbit anti-sheep IgGs) were purchased from Miles Laboratories (Elkhart, IN).

Immunofluorescence: Cells on coverslips were processed for immunofluorescence as described (28). Cytoskeletons were processed as above except that the acetone-treatment was omitted. The first antibodies were used at a dilution of 1/100 for FV3 antibodies, 225 μ g/ml for antitubulin and 1/20 for anti-58,000-dalton antiserum. FITC-conjugated antibodies were used at dilutions ranging from 1/20 to 1/40. RITC-phalloidin was used at a concentration of 1 μ g/ml. Controls which showed no fluorescence were: uninfected cells (in the localization of FV3 antigens), coverslips on which preimmune sera were used as first antibodies, and coverslips treated with heterologous antibodies. Coverslips mounted on glass slides with Elvanol (DuPont Co., Wilmington, DE) were viewed in a Zeiss photomicroscope equipped with epifluorescence optics and photographs were taken on Kodak Tri-X pan film.

Electron Microscopy: Monolayers of cells were prepared for electron microscopy as described (11) with two modifications. The primary fixative (glutaraldehyde) contained 0.4% tannic acid and the sections were stained with Reynold's lead citrate. Pellets of cytoskeletons were washed with PHEM (60 mM piperazine diethylsulfonic acid, 25 mM N-2-hydroxy-ethylpiperazine N1-2-ethanesulfonic acid, 10 mM EGTA, and 2 mM MgCl₂, pH 6.9) buffer (40) and fixed in 2.5% glutaraldehyde and 0.4% tannic acid made up in PHEM buffer. The remainder of the procedure was as described previously (14).

RESULTS

The morphological and biochemical alterations in FV3-infected BHK and FHM cells under a variety of conditions of infection have been described (9, 15, 33, 34). To identify stages in virus replication under the conditions of infection used here, and to provide background information for the subsequent time-course studies on cytoskeletal alterations, we first studied the sequence of FV3 development. FHM and BHK cells were fixed at various times after infection and examined by indirect immunofluorescence with FV3-antibodies and by electron microscopy. In FHM cells, virus assembly sites were first detected at 4 h after infection as distinct fluorescent bodies in the

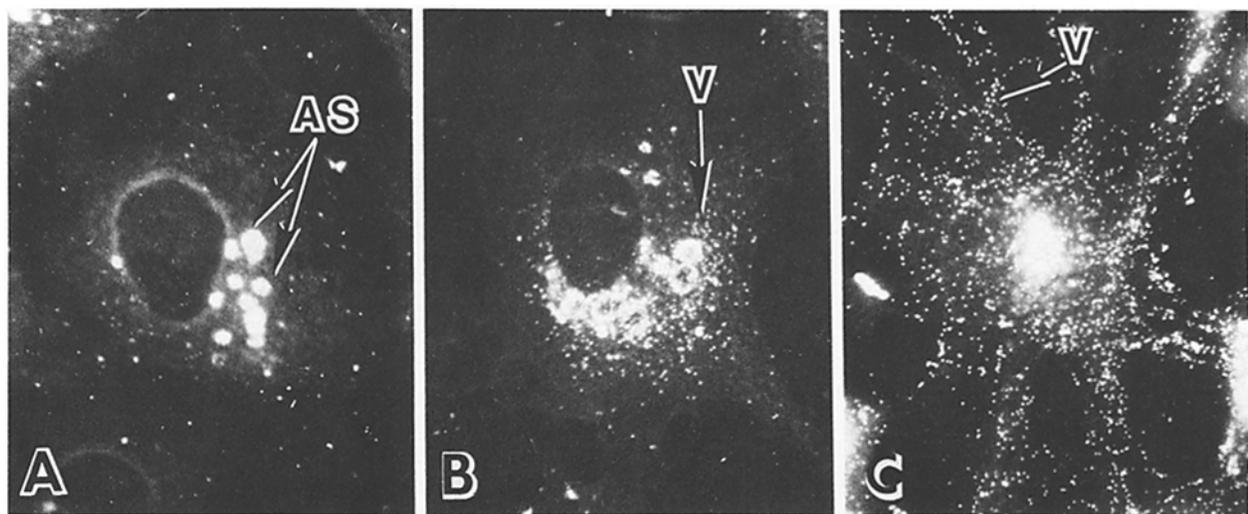


FIGURE 1 Indirect immunofluorescence with FV3-antibodies on FV3-infected FHM cells. (A) 4 h postinfection, (B) 6 h postinfection, and (C) 8 h postinfection. (AS) Virus assembly sites. (N) Nucleus. (V) Virions. $\times 1,100$.

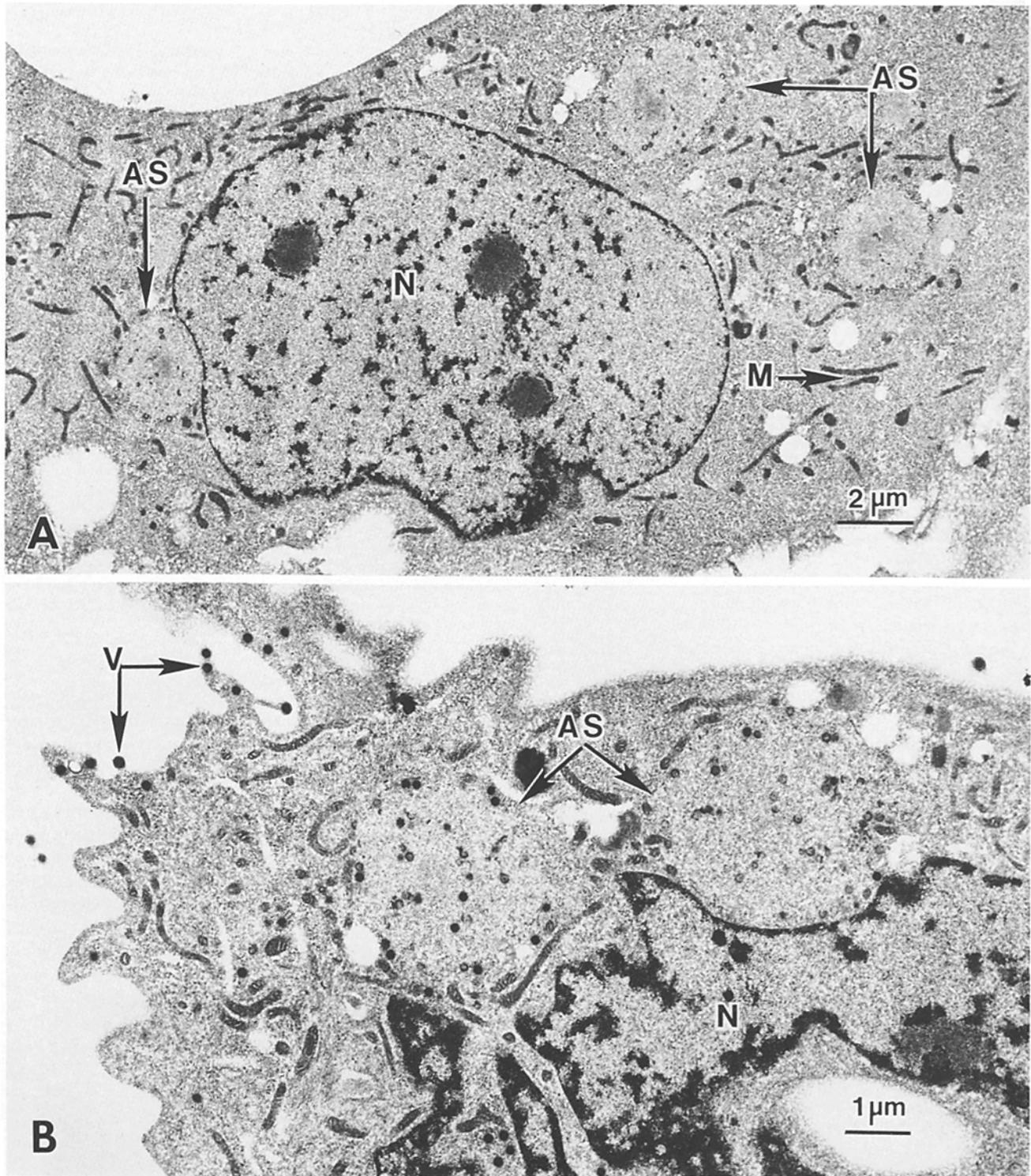


FIGURE 2 Electron micrographs of FV3-infected FHM cells at 4 h postinfection (A) and 6 h postinfection (B). The cells were cut parallel to the substrate. Abbreviations as in Fig. 1. (M) Mitochondria. (A) $\times 6,500$. (B) $\times 11,000$.

cytoplasm near the nucleus (Fig. 1A). Electron micrographs of infected cells at 4 h also revealed virus assembly sites (Fig. 2A). The sites had lower electron density than the surrounding cytoplasm, were devoid of cellular structures (e.g., organelles, cytoskeletal filaments, ribosomes), and contained assembling virions. That these sites correspond to the fluorescent bodies in Fig. 1A was further established by immunoperoxidase labeling and electron microscopy using the method of Graham and

Karnovsky (18). The peroxidase reaction product was localized within the assembly sites (data not shown). At 6 h after infection (Fig. 1B), there were many fluorescent particles at the periphery of the assembly sites and elsewhere in the cytoplasm. These presumably represent virus particles. In the corresponding electron micrographs (Fig. 2B), dense virus particles were seen in the assembly sites, in the adjacent cytoplasm and at the cell surface. At later times after infection, there was

a progressive increase in the abundance of virus particles (Fig. 1 C). In BHK cells the sequence of infection was similar, except it was slower by ~2 h. Thus, in BHK cells, the assembly sites formed at 6 h and mature virions appeared at 8 h.

Integration of Assembly Sites with Cytoskeleton

The juxtannuclear location of the assembly sites led to the suspicion that the viral components are anchored at that site by a component of the cytoskeleton. To verify this, monolayers of FHM cells were infected with the virus, treated with 0.15% Triton X-100, and processed for immunofluorescence microscopy using FV3 antibodies and electron microscopy. In cytoskeletons prepared from 4 h-infected cells, immunofluorescence analysis showed the retention of assembly sites (Fig. 3 A). The cytoskeletons of 6 h infected cells showed the association of assembly sites and some mature virions with the cytoskeleton (Fig. 3 B). An electron microscopic examination of sections of pellets of cytoskeletons confirmed the above observations (Fig. 4 A). The assembly sites in cytoskeletons, when examined at higher magnifications, revealed filaments (diameter 9–11 nm) embedded at the periphery of the assembly sites (Fig. 4 B) and contiguous with the cytoskeletal network.

Substructure of the Assembly Site

In earlier studies, the substructure of the assembly site was not clearly resolved but it was suggested that the site is composed of short, irregular filaments (9). In the present study, we found several well-spread assembly sites in cytoskeleton preparations in which the substructure was better resolved (Fig. 4 C). The site is composed of three components, a network of filaments measuring 2–3 nm in diameter, patches of electron-dense material, and virions at various stages of maturation.

The filaments are much thinner than microtubules (25 nm), intermediate filaments (9 to 11 nm), and microfilaments (6 to 8 nm) but could represent the filaments of the “microtrabecular lattice” (40). Another possibility is that the filaments represent viral DNA. This idea is supported by the correspondence of the diameter of the filaments (2–3 nm) with that of DNA (3) observed here, and earlier demonstrations of viral DNA in the assembly sites by feulgen staining (33), light microscope autoradiography (34), and electron microscope autoradiography (14). The electron-dense material presumably represents aggregates of viral protein. That the viral proteins are sequestered at the assembly sites was borne out by immunofluorescence studies described in this study and in earlier studies (33, 36).

Alterations in the Organization of Microtubules, Intermediate Filaments, and Microfilaments in Virus-infected Cells

We compared the organization of all three filaments in uninfected and infected BHK and FHM cells by immunofluorescence and electron microscopy. We illustrate the results of the immunofluorescence studies with BHK cells and electron microscopic studies with FHM cells. BHK cells are two- to threefold larger and flatter than FHM cells and therefore provide better images of the cytoskeleton at the light microscope level. FHM cells on the other hand, provide better electron micrographs in which larger areas of the cells can be observed at higher magnification. The morphological changes due to virus infection are similar in both cell types except that they are delayed by ~2 h in BHK cells.

When the organization of microtubules was studied in BHK cells by immunofluorescence using tubulin antibodies, the pattern observed was typical of normal cells (4). The microtu-

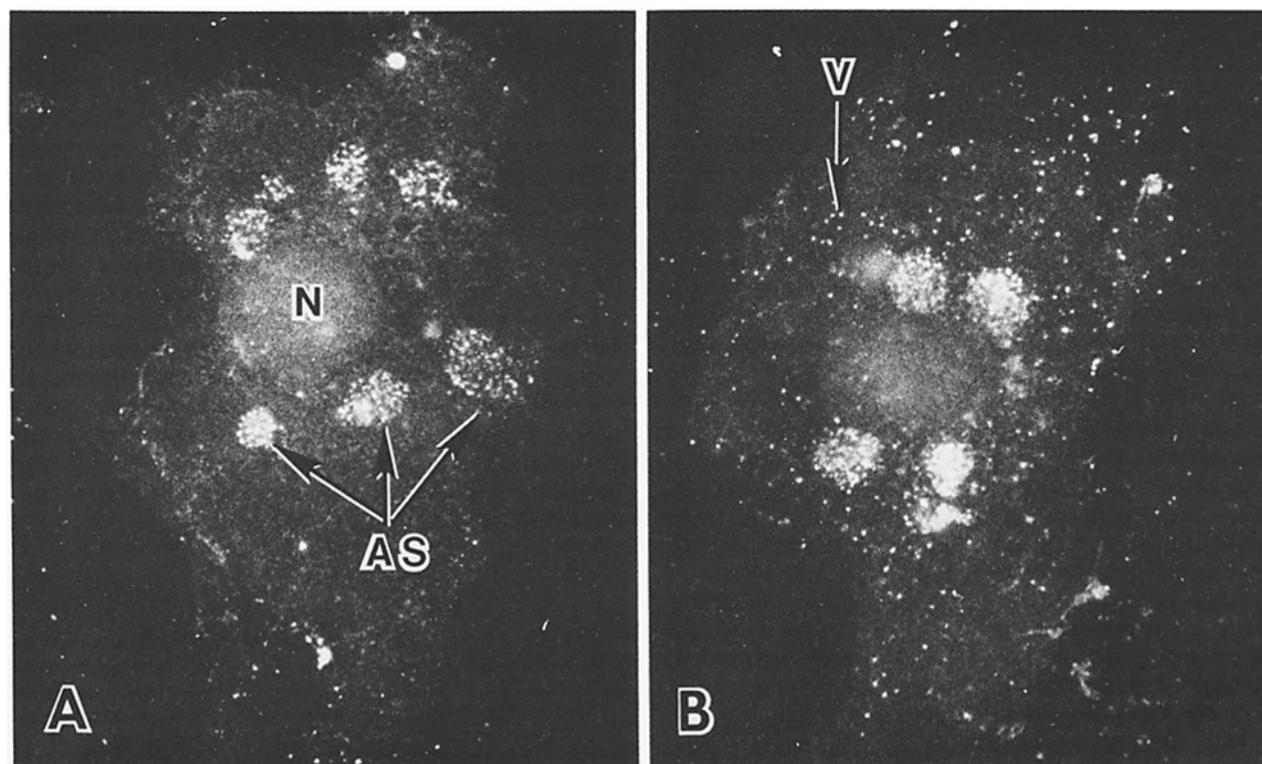


FIGURE 3 Indirect immunofluorescence with an FV3-monoclonal antibody on cytoskeletons prepared from FHM cells infected with FV3 for 4 h (A) and 6 h (B). Abbreviations as in Fig. 1. $\times 1,760$.

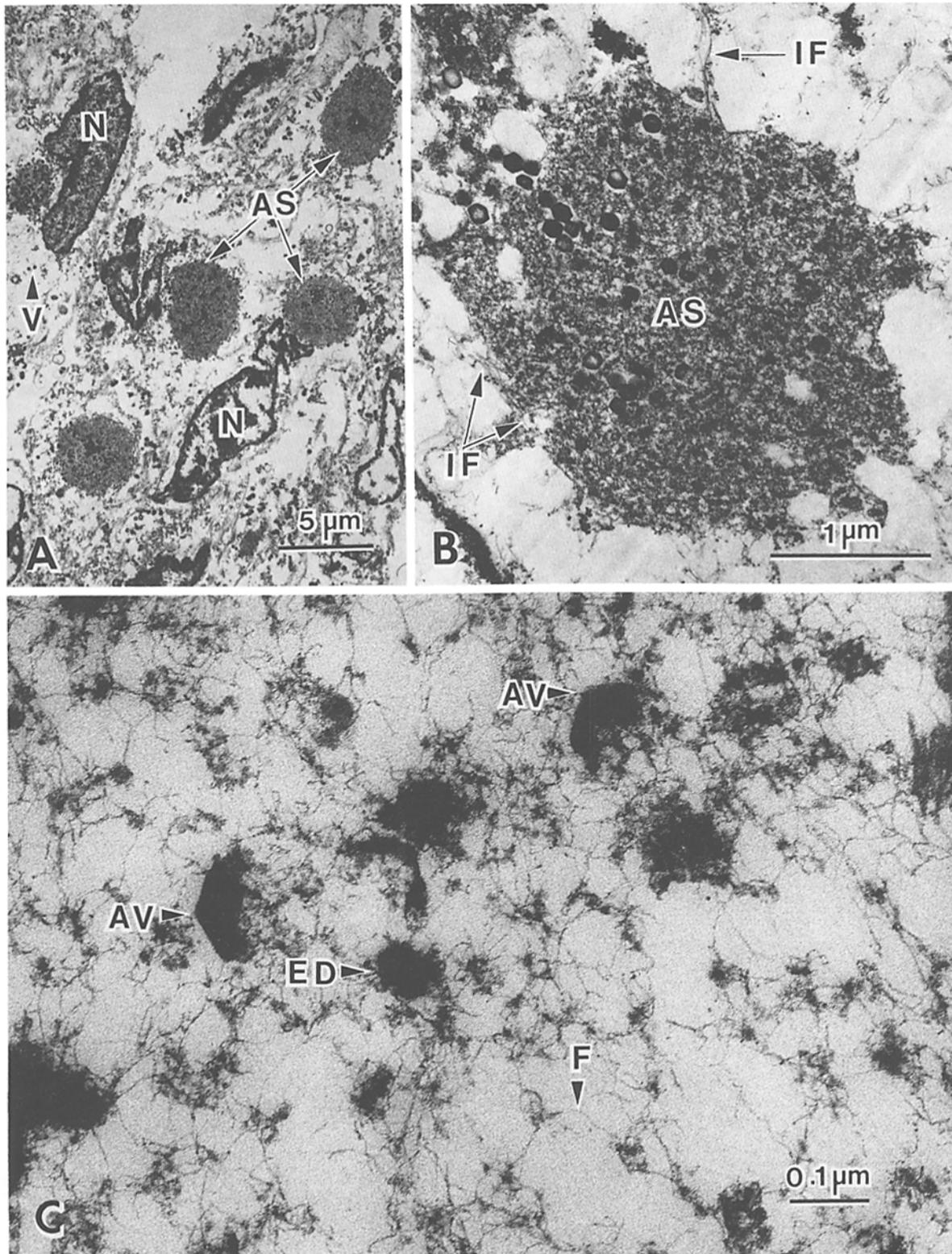


FIGURE 4 (A) Electron micrograph of a section through a pellet of cytoskeleton prepared from FHM cells infected with FV3 for 6 h. (B) An assembly site in the cytoskeleton shown at higher magnification. Note the intermediate filaments (*IF*) at the periphery of the assembly sites. (C) Substructure of the assembly site in cytoskeletons of 4 h-infected FHM cells. (*AV*) Assembling virions. (*ED*) Electron-dense material. (*F*) Filaments. Other abbreviations as in Fig. 1. (A) $\times 3,000$. (B) $\times 26,500$. (C) $\times 130,000$.

bules radiated from areas near the nucleus and formed networks in the cytoplasm (Fig. 5A). The same pattern prevailed in virus-infected cells until 5–6 h after infection. At 6 h, or at a time when the virus assembly sites begin to appear in these

cells, the number of microtubules decreased, and the focal points from which they radiated were more clearly seen (Fig. 5B). At 10 h after infection (Fig. 5C), there was a further reduction in the number of microtubules and some microtu-

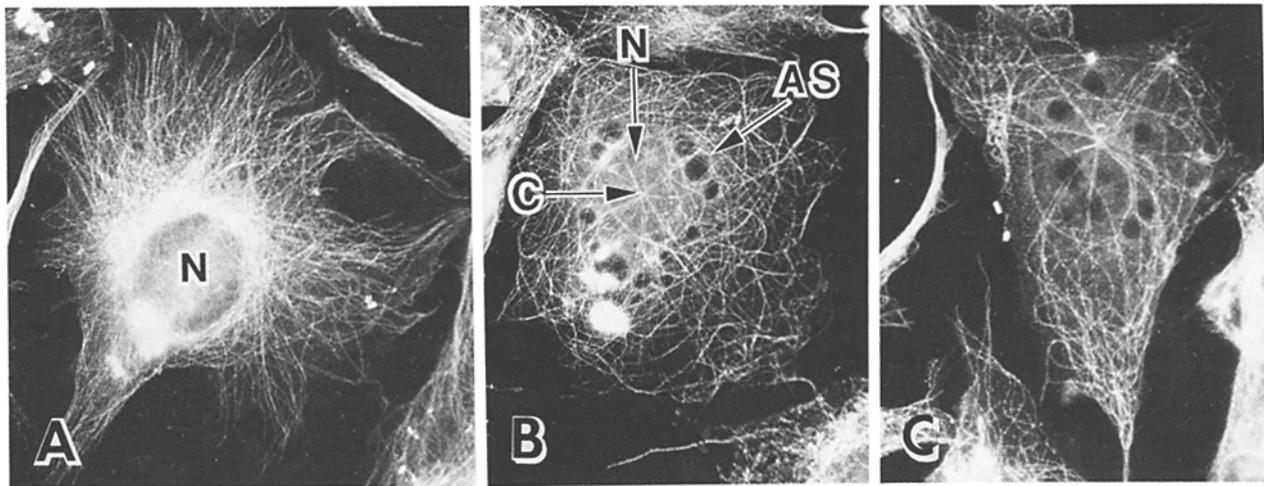


FIGURE 5 Indirect immunofluorescence with tubulin antibodies on BHK cells. (A) Uninfected cell, (B) FV3 infected cell at 6 h, and (C) 10 h. (Label C) Focal point. Other abbreviations as in Fig. 1. $\times 700$.

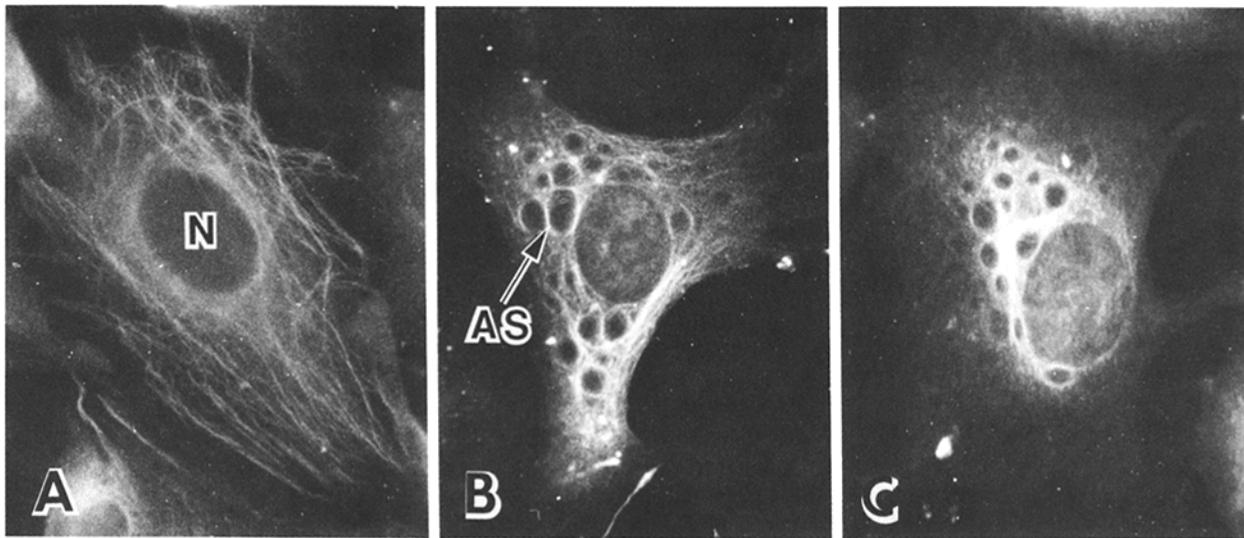


FIGURE 6 Indirect immunofluorescence with 58,000-dalton (vimentin) antibodies on BHK cells. (A) Uninfected cells, (B) cells infected with FV3 for 6 h and (C) for 8 h. Abbreviations as in Fig. 1. $\times 700$.

bules radiating from the central focus were seen to traverse in the vicinity of putative assembly sites. Electron micrographs of infected cells were less informative; however, the locations of centrioles between virus assembly sites and the presence of bundles of microtubules at the periphery of assembly sites were observed in some electron micrographs.

The organization of intermediate filaments in uninfected and infected cells was compared by immunofluorescence studies using antibodies to the 58,000-dalton intermediate filament protein (vimentin). In uninfected cells, the pattern resembled that seen in normal cells (22, 29). The filaments emanated from the perinuclear region and extended into the cytoplasm (Fig. 6A). In infected cells, we first saw changes in the organization of intermediate filaments at 6 h. The filaments retracted from the cell periphery and outlined certain discrete bodies in the cytoplasm (Fig. 6B). At 8 h, all of the fluorescence due to intermediate filaments was concentrated around the discrete bodies (Fig. 6C). We also observed the changes in the organization of intermediate filaments in electron micrographs. We saw bundles of intermediate filaments surrounding each virus

assembly site at earlier (Fig. 7A) and later (Fig. 7B) times after infection.

To compare the organization of microfilaments in uninfected and infected cells, we subjected the cells to immunofluorescence using RITC-conjugated-phalloidin (47). The uninfected cells showed a few thick cables (stress fibers) and many finer filaments running across the cell (Fig. 8A). In infected cells at the time of formation of the assembly sites, stress fibers were no longer seen, thin filaments persisted and the entire cell exhibited particulate fluorescence (Fig. 8B). The periphery of assembly sites showed diffuse fluorescence (Fig. 8B). At later times after infection, the cell surface contained many fluorescent projections (Fig. 8C). We did not detect these either in uninfected cells or in infected cells at earlier times, and we presume them to be cell surface specializations formed for virus release. Electron micrographs of infected cells at this time also revealed many viruses budding from cell surface projections either singly or in groups (Fig. 9). We saw bundles of microfilaments lining the plasma membrane and extending into the projections.

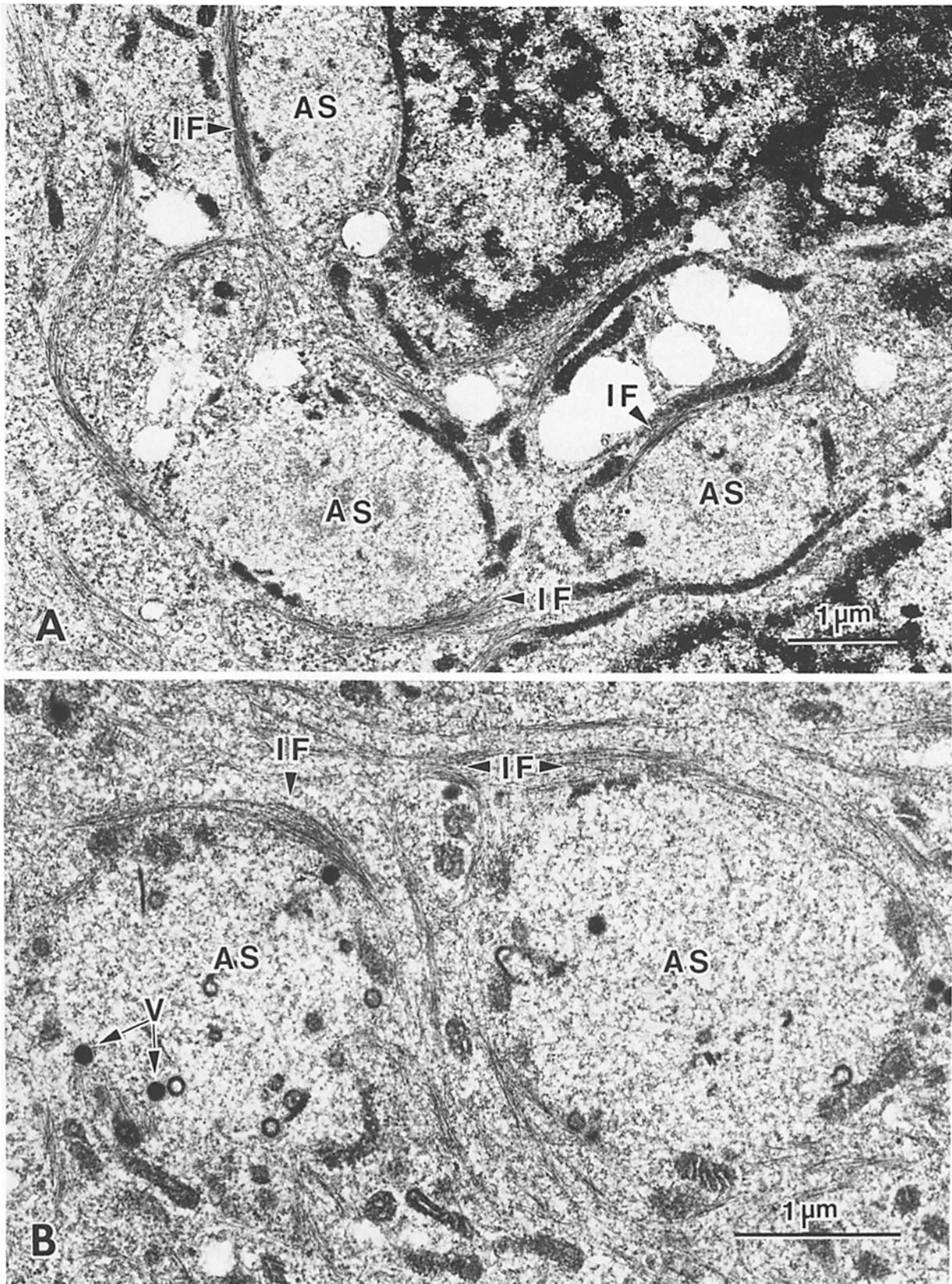


FIGURE 7 Electron micrographs of FV3-infected FHM cells at 4 h (A) and at 6 h (B). Note the bundles of intermediate filaments (IF) surrounding the virus assembly sites (AS). (A) $\times 21,000$. (B) $\times 28,500$.

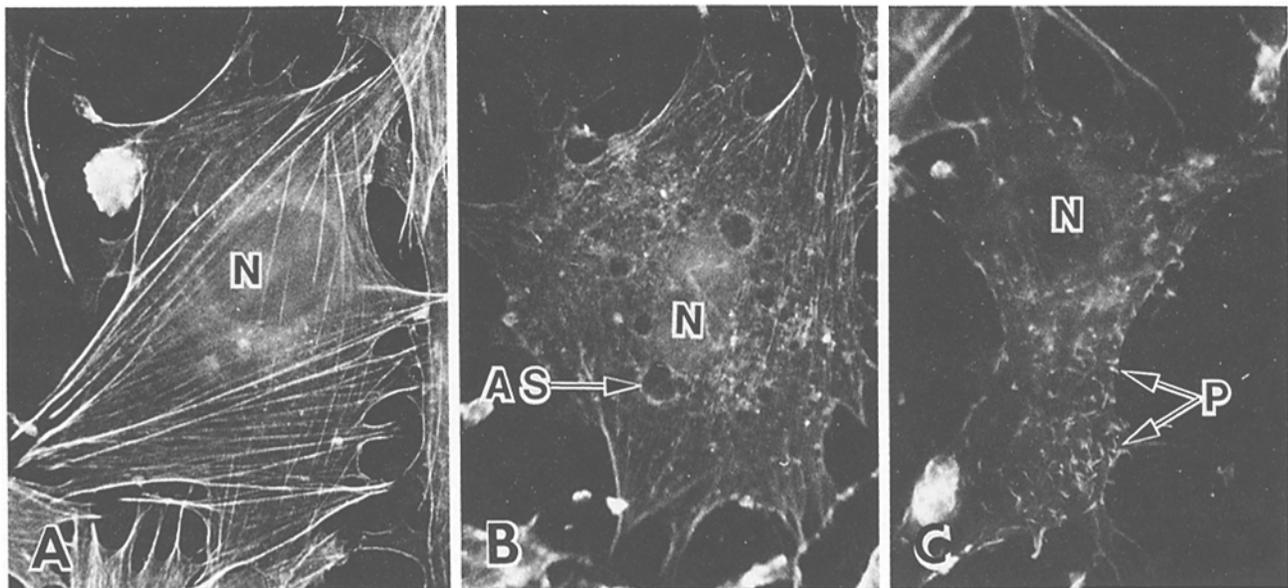


FIGURE 8 Immunofluorescence with rhodamine-conjugated-phalloidin on BHK cells. (A) Uninfected cells, (B) FV3-infected cells at 6 h, and (C) at 10 h. (P) Cell surface projections. Other abbreviations as in Fig. 1. $\times 750$.

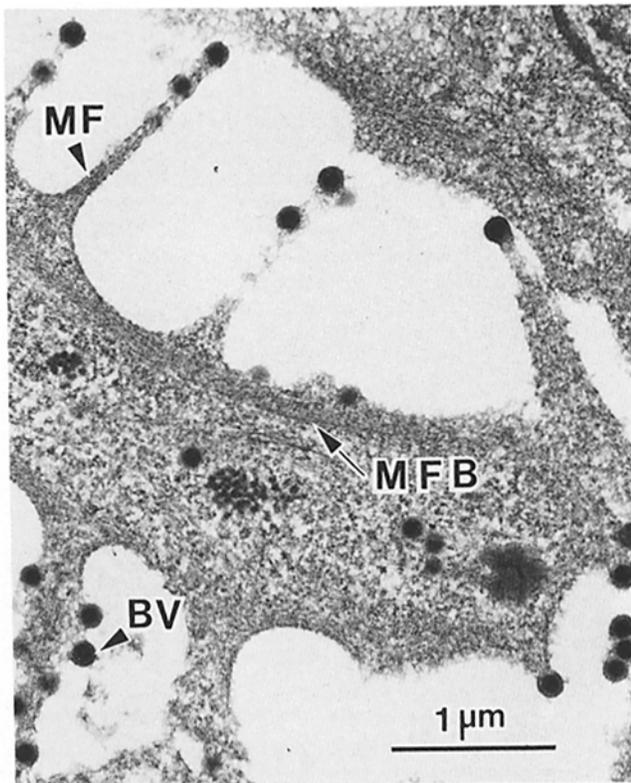


FIGURE 9 Electron micrograph of a portion of FHM cell infected with FV3 for 8 h. (MFB) Microfilament bundles. (MF) Microfilaments. (BV) Budding virions. $\times 21,500$.

Finally, to determine if the observed cytoskeletal alterations are due to the inhibition of host protein synthesis by the virus, we treated BHK cells with heat-inactivated FV3 (14) and studied the organization of all three classes of filaments by immunofluorescence. Heat-inactivated FV3 inhibits host protein synthesis just as efficiently as the normal virus but does not replicate (15). An examination of heated FV3-treated cells

showed none of the cytoskeletal changes observed in FV3-infected cells (data not shown), suggesting that the alterations are not due to inhibition of host protein synthesis.

DISCUSSION

Our results, taken together with those obtained by others (9, 14, 33, 34, 36), establish that FV3 assembles in distinct sites in the cytoplasm and that the sites contain viral genomes and viral proteins but no cellular structures (organelles, cytoskeletal filaments, ribosomes). In addition, the occurrence of discrete virus assembly sites in infected cells enabled us to demonstrate the intimate association of these sites with the cytoskeleton and to correlate cytoskeletal alterations with virus assembly. We now discuss the changes in all three classes of cytoskeletal filaments as they relate to virus development.

The organization of microtubules is strikingly different in FV3-infected cells compared to uninfected cells. In infected cells, beginning with the formation of the assembly sites, there is a progressive decrease in the number of microtubules radiating from the centrioles to the cell periphery. Late in infection, the few microtubules associated with centrioles are seen near the viral assembly sites. The precise role of these changes in microtubules in virus assembly is not clear. However, the preservation of microtubules adjacent to the assembly sites may have some significance in virus assembly. In normal cells, microtubules have been implicated in a variety of functions including the regulation of the location and movement of intracellular organelles (2). In FV3-infected cells, it is possible that the microtubules either independently, or in conjunction with other filaments (see below) are involved in the maintenance of virus assembly sites at a perinuclear location. Other functions of microtubules that may be pertinent to FV3 assembly are the movement of organelles and ribosomes (45) away from the assembly sites and the transport of viral components and viruses. At present, we have no evidence for such additional roles.

Intermediate filaments show a dramatic reorganization in FV3-infected cells. At a time when virus assembly sites just

begin to form, the filaments retract from the cell periphery and surround them. At later times, all of the intermediate filaments are found around the assembly sites. The reorganization of intermediate filaments could be explained in two ways. First, it is a secondary change brought about by the disruption of microtubules. It has been shown in a variety of normal cells that intermediate filaments are intimately associated with microtubules (12) and that the disruption of the latter by agents such as colchicine causes a retraction of the intermediate filaments to a perinuclear location forming juxtannuclear caps (44) or rings (22). The changes observed in FV3-infected cells resemble those seen in colchicine-treated normal cells in that both exhibit a disorganization of microtubules and a reorganization of the intermediate filaments at a perinuclear location. However, in FV3-infected cells, the intermediate filaments retract to surround the viral assembly sites which are located near the nucleus. This leads to a second hypothesis to explain the reorganization of intermediate filaments in FV3-infected cells, i.e., the filaments have a role in virus assembly. In normal cells, intermediate filaments exist in a polymerized state in most or all of the cell cycle and are highly insoluble in detergents (29). These observations suggested that the filaments have a structural role, perhaps in positioning the cell organelles at their proper locations (29). In FV3-infected cells, a similar role for the filaments can be envisioned in the formation and maintenance of virus assembly sites. By surrounding the viral genomes and proteins, the filaments might segregate them from other cellular structures and anchor them at fixed sites relative to the nucleus. This process may also involve microtubules. Such localization of viral components might facilitate an ordered assembly of the virus. The perinuclear location of the assembly sites may also be important since viral genomes are synthesized in the nucleus and transported to the assembly sites for the production of virions (14, 17).

In another study, an aggregation of intermediate filaments adjacent to the nucleus has been seen in poliovirus-infected cells by electron microscopy (31). A perinuclear aggregation of intermediate filaments was also noted in RSV-transformed cells but in the absence of detectable changes in the microtubules (1). In this case, it was believed that the phenomenon is due to the severance of molecular linkages between the two types of filaments by the viral transforming protein pp60^{src}. A disruption, rather than a reorganization, of intermediate filaments was found in reovirus-infected cells (43).

Microfilaments showed pronounced changes in their distribution in FV3-infected cells. In uninfected cells, actin is organized into thick bundles (stress fibers) and thin filaments. In the infected cells, at the time of formation of virus assembly sites, stress fibers disappeared and thin filaments persisted but were excluded from virus assembly sites. The lack or diminution of stress fibers has been reported in a variety of cells transformed with RNA or DNA viruses (13, 23, 24, 47). In these cases, the phenomenon is believed to be due to a reorganization of actin causing changes in cell shape and cell adhesion associated with the transformed state. Since FV3-infected cells also exhibit changes in cell shape and adhesion (25, 33), the observed alterations in microfilaments may be related to these rather than to virus assembly. However, the distribution of actin at later stages of infection is suggestive of a role for actin in the release of virus from the cell. Late in infection, the cells had many actin-containing projections at their surfaces which were not observed in uninfected cells or in cells at earlier times of infection. The projections most likely

represent cell surface specializations (blebs, microvilli, and ruffles) through which the virus emerges. A precedent for this exists in poxvirus-infected cells in which "specialized" microvilli form *de novo* in response to virus maturation (20). However, in poxvirus infected cells, no other changes in microfilament organization were detected. Thus, FV3-infected cells are unusual in showing disruption of stress fibers as in virus-transformed cells and a reorganization of actin at the cell surface as in poxvirus-infected cells. It is possible that the actin of the disrupted stress fibers is mobilized later to construct cell surface structures for virus release.

In summary, we have provided the first example of a virus-infected cells in which all three cytoskeletal filaments show profound changes in organization. The results presented here provide a basis to define events at the molecular level that are responsible for these alterations, and to analyze the significance of these alterations in virus development. We have begun biochemical studies to measure the qualitative and quantitative changes in filament proteins (tubulin, vimentin, and actin) in infected cells to understand their disorganization and reorganization. We are also examining virus growth in cells treated with agents that depolymerize the filaments or modify their organization (colchicine, vinblastine, taxol, cytochalasin, phalloidin, etc.), as well as filament organization in cells in which virus maturation is inhibited by chemical agents (e.g., azacytidine) or temperature-sensitive defects. These studies may eventually provide insights not only into the role of the cytoskeleton in virus metabolism but also into the processes involved in the turnover and organizational changes of the filaments in normal cells.

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