Frog Virus 3 Replication: Electron Microscope Observations on the Sequence of Infection in Chick Embryo Fibroblasts

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SUMMARY

The replication of frog virus 3 in primary chick embryo fibroblasts has been studied by examination of thin sections with the electron microscope and the assay of infectious virus. Uptake of frog virus 3 by the cells was observed to occur by pinocytosis and this may be the method of entry. Early in infection (1 h p.i.) marked margination of the nuclear chromatin occurred and the chromatin remained in this condition throughout the infection. Foci of infection were first detected in the cytoplasm of cells 24 h p.i. when production of infectious virus commenced. These foci appeared as electron translucent areas containing fine grains, surrounded by degenerate mitochondria. The foci usually contained virus particles. At this time budding of virus particles at the plasma membrane occurred. Later in infection at 36 and 48 h p.i. large numbers of virus particles were detected in the cytoplasm of cells either scattered loosely throughout the cell, arranged as clusters or in paracrystalline arrays. Extensive budding at the plasma membrane then took place. Virus particles were detected in the nucleus of the cells at these late stages and it is possible that the virus may infect and replicate at this site. Throughout the productive stages of infection aberrant forms of the virus, namely particles devoid of cores, incompletely assembled particles and elongated bacilliform particles were noticed.

INTRODUCTION

Frog virus 3 is a large icosahedral DNA virus which is apparently assembled in the cytoplasm of cells (Granoff, 1969; Kelly & Robertson, 1973). The virus will replicate in cell lines derived from mammalian, avian, piscine, and amphibian species (Granoff, 1969; Gravell & Granoff, 1970). Electron microscope studies of the sequential development of frog virus 3 replication has been confined mainly to mammalian cells, notably BHK 21/13 cells (Bingen-Brendel, Tripier & Kirn, 1971), though piscine cells, particularly fat head minnow (FHM) cells infected with the serologically related frog virus 1 have been examined in intermediate and late stages of infection by Darlington, Granoff & Breeze (1966). Primary chick embryo fibroblasts have been shown to readily permit infection by frog virus 3 (Gravell & Granoff, 1970; D. C. Kelly & M. G. Fenner, unpublished observations) though as yet no detailed electron microscope study of the infection process in these cells has been reported. Darlington et al. (1966) state that the terminal stages of infection of FHM cells resemble those found in chick embryo fibroblast cells infected with frog virus 1 and frog virus 3. Frog virus 3 replicates more slowly in chick cells than in BHK or FHM cells (Gravell & Granoff, 1970) and assuming that the various morphological stages in infection may be temporarily spaced apart, the cells appeared suitable to delineate the sequence of infection.
Fig. 1. Time course of the production of infectious frog virus 3 in primary chick embryo fibroblasts.

The study also facilitated a comparison of the infection process of frog virus 3 with that of other icosahedral cytoplasmic deoxyriboviruses (ICDVs) such as the insect pathogenic iridescent viruses (Kelly & Robertson, 1973) which have been studied in this laboratory (Kelly & Tinsley, 1974) as well as the interaction of frog virus 3 in other cell systems reported by Darlington et al. (1966) and Bingen-Brendel et al. (1971).

METHODS

Frog virus 3 was grown and titrated in primary chick embryo fibroblast cells at 28 °C as previously described (Kelly & Avery, 1974). Chick embryo fibroblasts were supplied by the Department of Biological Sciences at the University of Warwick. In this study the cells were allowed to grow to confluency at 37 °C in Falcon 25 cm² medical flasks. The cells were then maintained at 28 °C for about 12 h. The cells were infected by decanting the growth medium and allowing the virus (1 ml inoculum, 10 p.f.u./cell) to adsorb at 22 °C for 1 h. The monolayer was then washed twice with medium and 5 ml of medium was added to each monolayer. The infection was allowed to proceed at 28 °C. Samples were taken 1, 2, 3, 5, 8, 12, 24, 36 and 48 h after infection. Electron microscopy and the processing of samples were performed as previously described (Kelly & Tinsley, 1974).

RESULTS

Time course of production of virus

The overall pattern of replication of frog virus 3 in which cells was determined by plaque assaying sonicated material derived from cell sheets and the medium. The results are presented in Fig. 1. Infectious virus was produced 24 h post infection (p.i.) at a level above residual inoculum values. The growth curve approximates to a one step cycle of growth.
Fig. 2. An uninfected primary chick embryo fibroblast.

**Uninfected cell morphology**

Uninfected or mock-infected chick embryo fibroblasts typically contained nuclei in which the chromatin was condensed in one or two discrete central areas; ribosomes were plentiful and many were associated with endoplasmic reticula; the mitochondria appeared normal. Protrusions at the plasma membrane were frequently seen. No virus particles were detected in the cells (Fig. 2.).

**Early stages in infection**

1 and 2 h after infection virus particles were observed in close proximity to the plasma membrane, partially engulfed by the plasma membrane, in cytoplasmic vacuoles, and naked in the cytoplasm as shown in Fig. 3. This suggests that pinocytosis may play a role in the uptake of the virus particles.

At these early times the chromatin of the nuclei accumulated along the inner nuclear membrane in over 90% of the cells compared to 2 to 3% of the uninfected cells (Fig. 4).
Fig. 3. Apparent stages in the uptake and penetration of frog virus 3 (a) virus particle in juxtaposition to the plasma membrane; (b) (c), and (d) virus particles peripheral to the plasma membrane apparently linked to the plasma membrane and voiding the virus core; (e) loosely enveloped particles in intimate contact with the plasma membrane; (f) engulfment of virus particles; (g), (h), and (i) virus particles in cytoplasmic vacuoles; (j) a virus particle within a disintegrating cytoplasmic vacuole; (k) and (l) virus particles free in the cytoplasm. All observations made 1 h after infection.
Intermediate stages in infection

Three h after infection virus particles were no longer observed in cells, presumably because the input virus becomes uncoated and is no longer recognizable. No marked alteration in the cytopathology, other than the continued margination of the chromatin around the nucleus, occurred until 24 h had elapsed. Then areas of the cytoplasm which were probably destined as virus production sites were observed as electron translucent zones, with little fine structure other than fine granulation, surrounded by mitochondria (Fig. 5). These sites usually contained a few virus particles (Fig. 6), and occasionally areas containing abundant virus particles were seen (Fig. 7). Rarely small paracrystalline arrays of the virus were noticed. Viruses were budding at the plasma membrane at this initial productive stage (Fig. 10a), although few virus particles were present in the adjoining cytoplasm.
Late stages in infection

Thirty six and 48 h after infection over 90% of the cells contained virus particles and the virus was more abundant in individual cells than at 24 h. The particles were scattered throughout the cytoplasm, arranged discretely as clusters, or in paracrystalline array (Fig. 8). Areas resembling early foci contain an electron opaque network (Fig. 9) reminiscent of the virogenic stroma associated with nuclear polyhedrosis viruses (Harrap, 1972).

Extensive budding at the plasma membrane was observed (Fig. 10 and 11). The plasma membrane surrounding the virus particles became conspicuous, appearing thicker and staining intensely. This may be due to the increased angle of curvature of the membrane around the virus particle though it is unlikely because the neck of the goblet created late in budding does not exhibit this phenomenon (Fig. 11). It was common to see virus particles
fitting snugly at the end of long protrusions (Fig. 10b and 11c). Budding into cytoplasmic vacuoles was also observed and occasionally virus particles surrounded by one or two envelopes were seen in the cytoplasm (Fig. 12).

At both these late times about 10% of the nuclei contained virus particles (Fig. 13). The nuclei were intact, and apart from the enlargement of the perinuclear space, there appeared to be no breakdown of the nucleus. The mode of accumulation in the nucleus was not resolved. No virus particles were observed being transported across the nuclear membranes or within the perinuclear space, nor were enveloped virus particles seen in the nucleus. So although it is possible that cytoplasmically synthesized virus has entered the nucleus, it is also possible that the virus infects and replicates inefficiently within the nucleus. The latter interpretation is consistent with the occurrence of incompletely formed virus particles in the nucleus.

Throughout the productive stages of infection, and especially at late stages, aberrant forms of the virus were detected, particles devoid of cores, incompletely assembled particles, and elongated bacilliform particles (Fig. 14 and 15).
DISCUSSION

In many respects the morphogenesis of frog virus 3 in primary chick embryo fibroblasts is similar to that reported for the virus in BHK 21/13 cells by Bingen-Brendel et al. (1971) and frog virus 1 in FHM cells by Darlington et al. (1966), though the time sequence of events was extended and notable differences were observed.

Uptake of frog virus 3 was observed to occur by pinocytosis and apparently complete virus particles were also observed naked in the cytoplasm. Bingen-Brendel et al. (1971) suggest that uncoating of the virus occurs in the pinocytic vesicles and the genome is then released by rupture of the vesicles. Recently, Houts, Gravell & Granoff (1974) have also observed degradation of frog virus 3 in cytoplasmic vacuoles and they equate this with uncoating. This may occur but it appears incompatible with the observation of virus particles apparently intact in the cytoplasm an hour after infection. About 15% of the virus inoculum comprised enveloped particles (D. C. Kelly, unpublished observations), yet few enveloped particles were observed entering the cells. These particles may enter by fusion of the envelope surrounding the particle with the plasma membrane which is probably a rapid and, thus, infrequently observed phenomena. Such a process would give rise to intact
virus particles in the cytoplasm of cells. Houts et al. (1974) have made similar observations of intact virus particles free in the cytoplasm though they claim their inoculum (prepared by a similar method) to be free of enveloped virus particles and consider that the presence of intact virus particles is the result of direct penetration. The overall pattern of uptake of frog virus 3 resembles that of small iridescent viruses in insect cells (Kelly & Tinsley, 1974).

Early in infection pronounced margination of the nuclear chromatin was observed. This
Fig. 9. A cytoplasmic focus of frog virus 3 infection found 36 h after infection showing pathology typical of an early focus of infection except that it contains a network of electron dense material.

has also been observed by Darlington et al. (1966) and Campadelli-Fiume et al. (1973), and the latter authors correlate these morphological changes with the inhibition of host specific RNA and DNA synthesis in KB cells reported by Guir, Braunwald & Kirn (1971).

At intermediate stages of infection, the morphology of cytoplasmic foci of infection was identical to that reported by Bingen-Brendel et al. (1971) and Darlington et al. (1966), in which areas of reduced electron density containing finely granular material were surrounded by degenerate mitochondria. This cytopathology differs markedly to that found in iridescent virus cytoplasmic foci of infection where electron opaque areas devoid of mitochondria are seen (Kelly & Tinsley, 1974).

Later in infection, virus particles were arranged in crystals, which were probably organized as a three-dimensional structure, since the periodic differences in the intensity of staining
Fig. 10. Budding of frog virus 3 at the plasma membrane. (a) Virus particles observed 24 h after infection arranged at the plasma membrane (note that there are few virus particles in the adjacent cytoplasm); (b) virus particles budding profusely 36 h after infection.
Fig. 11. Detail of frog virus 3 budding at the plasma membrane. (a) A virus particle partly enveloped by the plasma membrane which shows pronounced staining where intimate contact with the particle occurs, and a particle enveloped by a darkly staining membrane which shows a tenuous link with the plasma membrane (arrowed); (b) A virus particle apparently in the terminal stages of budding (though it may be an enveloped particle entering by fusion): note that the envelope around the particle is densely stained whereas the neck of the goblet is not; (c) A virus particle located at the end of a cytoplasmic protrusion. Observations made 24 h after infection.

of adjacent virus particles in the crystals indicated that the section plane was different to the reticular plane. This is inconsistent with a two dimensional structure. The interparticle spacing was equal in the square and hexagonal lattices observed in Fig. 8a and b consistent with the arrangement of the virus particles in a face-centred cubic lattice with four virus particles per unit cell as deduced by Boulanger, Torpier & Rimsky (1973/74) for intranuclear adenovirus type 5 crystals.

Late in infection, cytoplasmic foci typical of early infection sites contained an electron dense fibrillar network. Although the phenomenon has not previously been reported in cell culture systems it has been observed with unidentified frog ICDVs in vivo in frog tumours (Lunger, 1969). Lunger (1969) has also noted the budding of frog ICDVs into cytoplasmic vacuoles, an occurrence previously not noted in cell cultures. This intracellular budding leads to the rare observation of multi-enveloped frog virus 3 particles in the cytoplasm of cells, a characteristic noted with an unrelated animal ICDV, African swine fever virus (Breese & DeBoer, 1966). Budding also occurs extensively at the plasma membrane. Bingen-Brendel et al. (1971) have compared the envelopment of frog virus 3 with the acquisition of a lipoprotein envelope by paramyxoviruses. This is not strictly analogous because the envelopment of frog virus 3 represents the addition of a lipid bilayer to an already completely
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Fig. 12. Cytoplasmic budding of frog virus 3 into a vacuole, showing unenveloped particles free in the cytoplasm and enveloped particles within the vacuole. Inset, multi-enveloped particles located in the cytoplasm. Observations made 24 h after infection.

assembled infectious icosahedral particle (Smith & McAuslan, 1969), whereas paramyxovirus envelopment represents the addition of an integral structural component of the virus particle to a randomly coiled nucleocapsid (Compans & Choppin, 1973). In the course of frog virus 3 budding at the surface membranes the lipid bilayer appears more electron opaque and thicker than the residual cell membrane. This may be created during the insertion or dislocation of virus structural polypeptides known to be specifically associated with the envelope (Tan & McAuslan, 1971). The opacity of the envelope is probably a transient phenomenon because enveloped virus particles which are apparently divorced from the cell, and also purified enveloped virus particles (D. C. Kelly, unpublished observations), lack this opacity.

Nuclear involvement in frog virus 3 replication was noticed late in infection. A similar observation was made by Darlington et al. (1966) on frog virus 1 infected FHM cells, though Bingen-Brendel et al. (1971) and Braunwald & Tripier (1974) categorically state that this is not observed in frog virus 3 infected BHK 21/13 cells. McAuslan & Smith (1968) have demonstrated, however, that frog virus 3 DNA is associated with the nucleus in BHK 21 cells. Nuclear involvement has not been observed in iridescent virus-infected cells (Kelly & Robertson, 1973; Kelly & Tinsley, 1974; D. C. Kelly & T. W. Tinsley, unpublished observations). If frog virus 3 can truly replicate in the cell nucleus then the term icosahedral
Fig. 13. Frog virus 3 particles located in the nucleus and cytoplasm, 48 h after infection.

Fig. 14. Aberrant forms of frog virus 3 located around a cytoplasmic focus of infection.
or polyhedral cytoplasmic deoxyribovirus frequently used to describe this virus (Granoff, 1969; Kelly & Robertson, 1973; Stoltz, 1973) appears inappropriate.

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REFERENCES


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