Evidence for incomplete replication of a penguin poxvirus in cells of mammalian origin

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The recent discovery of a novel poxvirus [penguinpox virus (PPV)] from Jackass penguins, Spheniscus demercus, has been characterized by enzyme restriction analysis and compared with that from other well-known avian poxviruses, including fowlpox (FPV), canarypox (CPV) and quailpox viruses (Kow, 1992). The distinctive restriction patterns suggest that these poxvirus isolates from penguins represent a new species of Avipoxvirus.

Poxviruses have been used for some years as vectors for the expression of foreign genes and the construction of recombinant vaccines. Initially, most of the attention was focused on vaccinia virus (VV), the prototype of the genus Orthopoxvirus (Fenner & Burnett, 1957), because of the extensive experience with VV as an immunizing agent and its well-defined molecular biology (Moss, 1990). However, the use of VV has always been contra-indicated in immunosuppressed persons, and there have been reports of severe and fatal disease in those infected with human immunodeficiency virus (HIV) (Redfield et al., 1987; Guillaume et al., 1991). An avirulent, highly attenuated VV strain, termed modified vaccinia Ankara (MVA), was developed by extensive serial passages in chicken embryo fibroblasts which resulted in lost capacity for productive infection in humans and other mammals. Despite the inability of MVA to produce infectious progeny, the expression of viral genes is unimpaired in human cells, and it has been evaluated for use as a safe expression vector (Sutter & Moss, 1992).

In contrast to VV, viruses of the Avipoxvirus genus have a restricted host-range and replicate productively only in susceptible avian species. There is evidence that avian viruses undergo abortive infection in mammalian cells (Taylor et al., 1988). In addition, it has been shown that inoculation of experimental animals with recombinant avipoxviruses can induce antibody to the product of the inserted gene, and protect them against challenge with the relevant pathogen, without any ill effect on the host (Taylor et al., 1991, 1992). For these reasons, recombinant avipoxviruses were suggested as potentially safer and effective vehicles for recombinant vaccines for human use (Baxby & Paoletti, 1992). CPV and FPV have been used successfully as recombinant vaccines to provide protective immunity in humans (Cadoz et al., 1992) and other mammals (Tartaglia et al., 1993). The poxvirus from the Jackass penguin, therefore, offers the potential of a novel alternative vaccine vector for use in terrestrial mammals. To assess this potential, a range of mammalian cell lines was infected with PPV, and replication was monitored by appraising viral DNA replication and using electron microscopy to determine the extent of virus morphogenesis.

Methods

Source of virus. PPV was isolated in this laboratory. A strain of CPV was kindly supplied by H. Mahnel, University of Munich, Germany.

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serum (FCS), containing penicillin (200 U)
minimum essential medium (MEM) supplemented with 10% foetal calf
HEF (human embryo fibroblasts). All cells were cultured in Eagle's
cervical carcinoma), MDBK (bovine kidney), RK-13 (rabbit kidney) or
fibroblasts (CEF), or one of the following lines of mammalian cells: CV-
Cell cultures.

Preparation of virus stocks. Virus stocks were prepared from the
chorio-allantoic membranes (CAMs) of embryonated hens’ eggs by a
modification of the method used by Joklik (1962). CAMs were harvested
4 days post-infection (p.i.) and placed in a bottle containing 1 ml per
membrane of a 3:1 mixture of McIlvain’s (MI) buffer (4 mM citric acid,
0.2 M Na₂HPO₄, 12H₂O, pH 7.4) and the organic solvent Arklo-X
(1,1,2-trichloro-1,2,2-trifluoroethane), and the suspension was shaken
vigorously for 2 min to disrupt the CAM tissue and release virus
particles. After brief centrifugation at 600 g, the supernate was harvested,
and the tissue pellet was once more treated to the extraction process.
Both supernates were pooled, and virus was semi-purified by differential
centrifugation through sucrose. Final virus pellets were suspended in TE
buffer (10 mM Tris, 1 mM EDTA, pH 9)

Cell cultures. Virus was cultured in either primary chick embryo
fibroblasts (CEF), or one of the following lines of mammalian cells: CV-
(1,1,2-trichloro-1,2,2-trifluoroethane), and the suspension was shaken
vigorously for 2 min to disrupt the CAM tissue and release virus
particles. After brief centrifugation at 600 g, the supernate was harvested,
and the tissue pellet was once more treated to the extraction process.
Both supernates were pooled, and virus was semi-purified by differential
centrifugation through sucrose. Final virus pellets were suspended in TE
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Staining for intracellular viral DNA. Hoechst’s 332558 fluo-
rescent DNA stain, as described by Russell et al. (1975), was used to
identify cytoplasmic DNA in infected cell monolayers at various times
p.i.

Extraction of DNA

(a) Viral DNA from infected CAMs. PPV particles in TE buffer (har-
vester from infected CAM tissue) were lysed by the addition of an
equal volume of 100 mM Tris buffer, pH 8.0, containing 4% sodium
N-laurylsarcosinate, 54% sucrose and 200 mM β-mercaptoethanol, for
10 min at room temperature. Proteinase K (Boehringer Mannheim) was
added to a concentration of 100 µg/ml, and the mixture incubated at
56 °C for 1.5 h. DNA was purified by repeated extractions with phenol
and chloroform–isoamyl alcohol (24:1), and precipitated with absolute
ethanol in the presence of 0.3 M sodium acetate at −20 °C.

(b) Total DNA from infected CV-1 cells. CV-1 monolayers were inocu-
lated with PPV at an m.o.i. of 5. The inoculum was removed after 1 h
and replaced with culture medium, and the cells were incubated at
37 °C for 3 days. The cells were removed from the culture flask and
suspended in lysis buffer (100 mM Tris, 100 mM NaCl, 5 mM EDTA,
1% SDS). Proteinase K was added to a final concentration of 100 µg/ml
and lysis was accomplished at 55 °C for 3 h. DNA was extracted from
the cell lysate with phenol–chloroform and purified by precipitation
with ethanol.

Endonuclease restriction of viral DNA. DNA extracted from
purified PPV propagated on CAMs, or from PPV-infected CV-1 cells, was
subjected to restriction digestion with PstI (Boehringer Mannheim), using
standard procedures, and electrophoresed in the presence of ethidium
bromide, on 0.8% agarose gel. Stained DNA was visualized by trans-
illumination with UV light.

Assays for infectious progeny virus. CV-1 cell monolayers in
25 cm² flasks were infected with PPV at an m.o.i. of 0.5, 0.2 or 0.1 p.f.u.
per cell; 1 h p.i. the inoculum was removed, the cell layer washed with
PBS and covered with MEM containing 4% FCS. The cell monolayer
from one flask was washed twice with PBS, removed by trypsinization,
concentrated in a microfuge, washed once in MI buffer, suspended in
400 ml of MI buffer and stored at −20 °C. This procedure was repeated
on separate monolayers taken at 1 h p.i., and then at 24 h intervals for up
to 5 days. Virus was released from the cells by four cycles of freezing
and thawing and cell debris was removed by low-speed centrifugation.
Tenfold dilutions of both culture medium and cell lysate from each flask
were titrated by plaque assay on the CAMs of 10-day-old chick embryos.

Parallel titrations were performed with cultures that had been exposed
to an inhibitor of DNA replication, cytosine arabinoside (Ara-C)
(40 µg/ml), in both virus inoculum and culture fluid.

Attempted passage of PPV in CV-1 or CEF cells. Cell
monolayers were inoculated with PPV at an m.o.i. of 5 p.f.u. per cell; 1 h
p.i. the inoculum was removed and the monolayer was washed twice with
PBS. The cell layers were covered with medium and returned to 37
°C for 5 days. Cell layers were removed from the culture flask by trypsin,
and lysed by three successive cycles of freezing and thawing. The lysate
was then used to inoculate fresh cell layers, one-tenth of the lysate being
retained for titration on CAMs. This procedure was repeated three times.

Electron microscopy of PPV-infected cells. CV-1 cell mono-
layers were infected with PPV at an m.o.i. of 5 p.f.u. per cell. After 72 h
the medium was removed and the cell layer washed three times with
phosphate buffer, pH 7.2 (PBS). The cells were removed from one culture
flask, suspended in a small volume of distilled water and subjected to four
cycles of freezing and thawing. Cell debris was removed by low-speed
centrifugation and the supernatant fluid was centrifuged at 19000
g for 20 min in a Beckman SW50.1 rotor. The pellet was negatively stained
with 1% phosphotungstic acid (pH 6.0), and examined in an Hitachi-600
electron microscope.

Ultra-thin sections were prepared of PPV-infected CV-1 cells or
primary CEF cells, and of PPV-infected CAMs, harvested at 72 h p.i. Cell
cultures were rinsed three times with PB, fixed in 2% glutaraldehyde for
2 h at 4 °C, post-fixed in 1% OsO₄ for 1 h at 4 °C, then dehydrated and
embedded in Spurr’s resin by conventional methods. Ultra-thin sections
were post-stained with uranyl acetate and lead citrate before examination
in the electron microscope.
Assessment of transient gene expression. The capacity for foreign gene expression in PPV-infected cells was assessed in a transient expression system (MacGregor & Caskey, 1989) using plasmids in which the reporter gene lacZ, which encodes β-galactosidase (β-Gal), was under the transcriptional control of the VV promoters, P11 (late) (plasmid pAL1), P7.5 (early/late) (plasmid p182) and P4b (late) (plasmid p4b) respectively. The plasmids were kindly supplied by M. Mackett, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, UK. In comparative control studies, CPV was used in the place of PPV.

Cell monolayers in 30 mm plates (approximately 10^6 cells per plate) were infected with either PPV or CPV at an m.o.i. of at least 5; 1 h p.i. the virus inoculum was removed and cells were transfected with 5 µg of one of the three plasmids using liposomes (DOTAP, Boehringer Mannheim) as per the manufacturer’s instructions. At 24, 30 and 48 h.p.i., culture medium was removed and cells covered with PBS containing the substrate X-Gal at a concentration of 1 mg/ml. After 18 h, β-Gal activity was determined by counting the number of blue cells (MacGregor et al., 1991). Experiments were also performed in the presence of 40 µg/ml Ara-C, in the culture medium, the transfection medium and the virus inoculum.

Results

Intracellular viral DNA

Fluorescent staining for DNA in PPV-infected CV-1 cells 48–72 h p.i. revealed discrete foci of bright fluorescence in the cell cytoplasm, indicating that replication of viral DNA had occurred. No fluorescence was seen in the cytoplasm of uninfected cells.

Total DNA was extracted from PPV-infected CV-1 cells, digested with PstI and electrophoresed in parallel with DNA from purified PPV virions harvested from infected CAM tissue (Fig. 1). In the cellular DNA (lane 3), bands were visible (against the smeared background of nuclear DNA) that matched those in the control PPV PstI restriction profile (lane 2). Viral DNA from residual inoculum would have been insufficient to account for their presence, and similar bands were not seen with DNA from uninfected cells (lane 4).

Assays for infectious progeny virus

CV-1 cells infected with PPV were disrupted at various time intervals p.i. and the lysates were assessed for the presence of infectious virus by titration on CAMs. Results are summarized in Table 1. In none of the assays was there a detectable increase in intracellular virus titre over 5 days, and the infectivity in eggs remained virtually unchanged from day 0 to day 5. Likewise, titration of extracts from CV-1 cells infected with PPV in the presence of Ara-C gave no change in

<table>
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<tr>
<th>Inoculum in CV-1 cells (p.f.u. per cell)</th>
<th>Titre (log_{10} p.f.u./ml) of PPV from cell lysate, titrated on CAMs</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>0.2</td>
<td>4.0</td>
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<td>3.4</td>
</tr>
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<td>0.1 (+Ara-C)</td>
<td>3.6</td>
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</table>

Table 1. Infectivity of PPV harvested from CV-1 cells

Fig. 2. (a) Negatively stained virion from PPV-infected CAM tissue, possessing characteristic morphology of a mature poxvirus. (b) Immature virions recovered from the cell lysate of PPV-infected CV-1 cells. Bars, 100 nm.
Fig. 3. The morphogenesis of PPV is similar in both chick embryo and CAM cells. (a) ‘Crescents’ and immature particles with condensing nucleoids in CAM cell cytoplasm. (b) Maturing particles in a CEF cell are elongated and contain biconcave cores. They become wrapped (enveloped) by membranes of distended cytoplasmic cisterna. (c) Mature poxvirus apparently budding from the plasma membrane of a CAM cell. (d) Intracytoplasmic inclusion in PPV-infected CEF cells contains enveloped virions within a matrix of lipid and fibrillar material. Bars, 300 nm. E, intracellular enveloped particle.

virus titre over 5 days. Titration of the culture fluid removed from infected cell layers also showed no change in virus titre. Since suspensions of PPV incubated at 37 °C for 5 days did not decline significantly in titre, it was assumed that the titres obtained from the infected cells or culture fluids resulted from residual inoculum virus.

Titrations on CAMs, performed after each of three sequential passages of PPV-infected CV-1 cells, showed no
detectable infectious virus after the first passage. Attempts to passage PPV in CEF cells were also unsuccessful.

**Electron microscopy of PPV-infected cells**

PPV particles harvested from CAMs (Fig. 2a) have characteristic poxvirus morphology. Similar intact virions were not found in cell lysates of PPV-infected CV-1 cells, but particles resembling immature forms of the virus were seen (Fig. 2b). These particles possessed an apparently intact core membrane, but the palisade layer and surface tubules were not present in the structured array usually associated with the mature virions recovered from CAM extracts.

The degree of PPV virus maturation within CV-1 cells was examined by thin-section electron microscopy and compared with that found in both CEF cells and CAMs of embryonated hens’ eggs. In cells of avian origin, the replication of PPV followed the same sequence as that reported for a variety of poxviruses including the FPV. Immature virus particles, which formed around cytoplasmic masses of granular material, originated as ‘crescents’ (Fig. 3a) that became closed to form double-shelled spherical particles enclosing apparently homogeneous matter of fine granularity. The inner shell then contracted, the virion shape changed from spherical to oblong, and the contents became condensed and differentiated into the typical biconcave core flanked by two electron-dense lateral bodies. These mature particles (Fig. 3b) became aligned against membranes of distended cytoplasmic vesicles, possibly Golgi in origin, and by budding through that membrane acquired an additional envelope. Enveloped progeny virions were released from the cell, presumably by fusion of the encompassing membrane vesicle with the plasma membrane. Occasionally, virions were seen apparently budding from the plasma membrane of CAM cells (Fig. 3c). Large membrane-bound inclusion bodies were seen in both CAM tissue and CEF cells infected with PPV. In CEF cells these appeared to originate from distended Golgi cisterna that became filled with a matrix of lipid and tubular structures (Fig. 3d). Virions within these
inclusion bodies possessed envelopes that were acquired, apparently, by budding through the surrounding membrane. Additional inclusion bodies containing no virus particles and filled with electron-dense proteinaceous material were also present in infected CAM cells. The sequence of maturation of PPV in CEF cells is diagrammatically illustrated in Fig. 4 (left), and compared with the incomplete maturation observed in the mammalian CV-1 cell line (Fig. 4, right).

In PPV-infected CV-1 cells, the appearance of ‘crescents’ and spherical immature virus particles in the cytoplasm (Fig. 5a) was identical to that observed in the avian cells. Cytoplasmic virus particles became oblong in shape, but their contents remained disorganized and heterogeneous in appearance (Fig. 5b, c). In the majority of cases, the core membrane did not become detached from the immature virus coat, and distinctive biconcave core structures or lateral bodies were rarely seen. At no stage were these partially mature virions observed to bud or acquire an extra envelope, either from cytoplasmic membranes or from the cell membrane. Infected cells eventually died leaving large numbers of incomplete poxvirus particles randomly distributed within the degenerate cytosol (Fig. 5d).

Fig. 5. Incomplete maturation of PPV in CV-1 cells. (a) Crescentic and spherical immature particles in the cell cytoplasm. (b, c) Particles become oblong in shape, but their contents remain amorphous and uncondensed. (d) Incomplete particles do not become enveloped, and remain scattered throughout the cytosol of the degenerating cell. Bars, 300 nm.
An unexpected and remarkable additional observation was the presence of ‘crescents’ and immature virus particles within the nuclei of a number of infected CEF cells (Fig. 6a–c). Serial sections through these cells confirmed that the intranuclear particles were assembled in situ, and did not result from intrusions of cytoplasm into the cell nuclei.

**Transient gene expression**

The degree of PPV maturation in the CV-1 mammalian cell line indicated that both early and late gene products were being translated. It was therefore pertinent to test whether PPV transcriptases would recognize early and late promoter sequences derived from VV. Each of the three β-Gal plasmids was transfected into CV-1 cells infected with PPV. All three promoters gave positive results, but the P11 promoter was found to be more efficient than the other late promoter (P4b) or the P7.5 promoter (data not shown). In further experiments, the plasmid containing the P11 promoter was used in mammalian cells that had been infected with PPV. Tests were performed in CV-1, Vero, MDBK, HeLa and RK-13 cell lines, as well as HEF cultures. CEF (avian) cells were used as a control.
abortive infection in the foreign host. PPV was able to gain vaccine vector for use in mammals is its ability to initiate an requirement for consideration of avipoxvirus as a potential chance infection of the natural host by the vaccine strain. A first marine habitat of penguins would limit the opportunity for vector for terrestrial mammals, PPV would have certain (unpublished data). As a candidate for utilization as a vaccine a bantam fowl) that occur in the same geographical area other avipoxviruses (one from a turtle-dove and the other from FPV and quailpox virus). It is also quite dissimilar from two distinct from other laboratory strains of avipoxvirus (CPV, [unpublished data]. As a candidate for utilization as a vaccine expression in HEF cells, but unlike the continuous cell lines, cells and none at all in RK-13 cells. There appeared to be good expression in HEF cells, but unlike the continuous cell lines, uninfected HEF cells exhibited a low background level of endogenous β-Gal. When comparative assays were performed with CPV, expression was less efficient than after infection with PPV in both avian and mammalian cells (Table 2).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>−Ara-C</th>
<th>+Ara-C</th>
<th>−Ara-C</th>
<th>+Ara-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>+ + + +</td>
<td>Neg.</td>
<td>+ +</td>
<td>ND</td>
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<tr>
<td>CV-1</td>
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<td>Vero</td>
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<tr>
<td>MDBK</td>
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<tr>
<td>HEF</td>
<td>+ +</td>
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Gene expression, in the absence or presence of Ara-C, was assessed after staining with X-Gal, and the levels of expression are summarized in Table 2. Strongly positive gene expression in CEF cells indicated the potential of PPV to replicate in alternative avian cells. In the mammalian cell lines, highest levels of β-Gal expression were observed in CV-1 cells where it was detected in up to 50% of cells and occurred only in the absence of Ara-C. Good expression was seen in Vero cells, while only poor expression was found in MDBK and HeLa cells and none at all in RK-13 cells. There appeared to be good expression in HEF cells, but unlike the continuous cell lines, uninfected HEF cells exhibited a low background level of endogenous β-Gal. When comparative assays were performed with CPV, expression was less efficient than after infection with PPV in both avian and mammalian cells (Table 2).

Discussion

The novel penguinpox virus described in this report has been shown (by endonuclease restriction analyses) to be distinct from other laboratory strains of avipoxvirus (CPV, FPV and qualipox virus). It is also quite dissimilar from two other avipoxviruses (one from a turtle-dove and the other from a bantam fowl) that occur in the same geographical area (unpublished data). As a candidate for utilization as a vaccine vector for terrestrial mammals, PPV would have certain advantages, not least of which is the fact that the coastal and marine habitat of penguins would limit the opportunity for chance infection of the natural host by the vaccine strain. A first requirement for consideration of avipoxvirus as a potential vaccine vector for use in mammals is its ability to initiate an abortive infection in the foreign host. PPV was able to gain entry into the simian CV-1 cell line, and replication of viral DNA was indicated by discrete pockets of fluorescent DNA in the cell cytoplasm. The fact that Pst I endonuclease digestion of DNA extracted from these infected CV-1 cells resulted in a typical PPV restriction profile provided supportive evidence for DNA replication.

The success of a virus vector in the expression of foreign genes is naturally dependent upon the efficient translation and processing of the gene products in the immunized host. Despite the evidence for replication of PPV DNA in the CV-1 cells, it was not possible to recover infectious progeny virus from lysed CV-1 cells, indicated by titration of lysates on CAMs of chick embryos over a period of 5 days. Attempted sequential passage of PPV in CV-1 cells was also unsuccessful up to three passages. Negative-stain electron microscopy of PPV-infected CV-1 cell lysates showed the presence of immature poxvirus particles, indicating that at least some of the structural proteins were being synthesized and assembled. Ultra-thin sections of the infected cells were therefore examined to assess the degree of morphogenesis of PPV in the mammalian cells. These studies confirmed that early stages of virus replication (crescent and spherical forms) occurred in a similar fashion to that seen in avian cells, but that virus assembly did not proceed to the formation of morphologically mature particles. Our observations were similar to those described by Somogyi et al. (1993) for FPV in Vero cells.

The development of PPV in either CEF or CAMs was similar to that described for other avian viruses including a turkey poxvirus (Cheville, 1966) and pigeonpox virus (Purcell et al., 1972) where mature virions possess a characteristic dumbbell-shaped core flanked by two electron-dense lateral bodies and surrounded by a coat. These mature particles acquire an extra envelope by budding into cytoplasmic vacuoles where they often accumulate to form large so-called inclusion bodies. In PPV-infected CV-1 cells, the most apparent deficiency in virus maturation was in the lack of organization of the internal core components. This may reflect inadequacies in the content of internal proteins, or in DNA packaging, although it was obviously not possible to make this determination on the basis of morphological appearance. The inefficient acquisition of lateral bodies and possible malfunction of the viral coats could account for the absence of association between the poxvirus structures and any cytoplasmic membranes, and consequently their inability to acquire envelopes by budding. While it is generally agreed that envelopment is required for externalization of poxvirus particles, and that both intracellular ‘naked’ particles as well as extracellular enveloped particles are infectious, the minimal degree of structural integrity required for poxviruses to initiate an infection is not known. The fact that no infectious virus could be demonstrated in lysates of infected CV-1 cells suggests that all of the observed immature virus structures were incapable of dissemination of infection. We are unable to explain the inability to passage PPV successfully in CEF cells,
despite the fact that progeny virions after one cycle of infection were apparently morphologically complete. It is possible that the processing of viral coat proteins in CEF cells is in some way inadequate to produce compatible receptor sites on the virion surface, but without specific protein analyses, such concepts are entirely speculative.

Of added interest to this study was the observation of crescentic and spherical viral particles in the nuclei of PPV-infected CV-1 cells. Within PPV-infected avian cells, these early immature core structures were confined exclusively to the cytoplasm. Arhelger & Randall (1964) observed both immature and mature forms of PPV in the nuclei of infected CAMs, but the origin and significance of intranuclear poxvirus particles has received little attention thus far. It was originally proposed that the viral crescents of VV consisted of membranes synthesized de novo within the cytoplasm by virally encoded enzymes (Stern & Dales, 1976), but a more recent report has proposed that they are derived from a membrane cisternum of the intermediate compartment (Sodeik et al., 1993). The assembly of PPV crescents within the nucleus of the cell (albeit in an unnatural host) poses new questions regarding their derivation, and indeed brings into question whether they are truly membranous in nature. This aspect is presently being investigated in our laboratory.

When replication of PPV was assessed under the control of the VV P11 late promoter, transient gene expression was confirmed in a range of mammalian cells, and in all cases expression was halted by the addition of Ara-C, in accordance with findings that indicate the overall high potency of the 4b promoter in both FPV- and VV-infected cells (Binns et al., 1989). The assembly of PPV crescents within the nucleus of the cell (albeit in an unnatural host) poses new questions regarding their derivation, and indeed brings into question whether they are truly membranous in nature. This aspect is presently being investigated in our laboratory.

References


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